

More conviction on HIV and AIDS

A thorough study of the death-rate among British haemophilia patients with or without HIV infection will, for most people, be sufficient proof that the infection leads to AIDS.

NOT much has recently been heard from those who hold that HIV has nothing or little to do with the causation of AIDS. The letter from Sarah C. Darby *et al.* on page 79 of this issue will be a further reason for discretion of their part. A group of epidemiologists has studied the total membership of the British National Haemophilia Register between 1977 and 1991, using it to tell the respective fates of those who were or were not infected with HIV during the period from 1979–86, when blood products in Britain and most other places were contaminated by HIV. The total of 1,227 people infected at that time amounts to roughly a fifth of the 6,287 people on the register. The most dramatic outcome of the study is that the death rate (from all causes) among those infected with HIV has been roughly ten times greater than among the uninfected, and that the death-rate among the infected is not influenced by the clinical severity of their haemophilia. The group estimates that 85 per cent of the deaths among the infected patients were caused by HIV.

This further evidence of the association of HIV with untimely death is important for several reasons. For one thing, it is the largest study of its kind yet carried out. Where statistical data are used to support claims of association, sheer numbers are important; close on 1,000 of those on the register have died since 1978, enough to make the interpretation statistically significant. It is also relevant that the numbers lost to the register through emigration from Britain and other unspecified causes is small at 3.5 per cent. Suggestions that the conclusions of the study are vitiated by the loss of data for a biased group of individuals, perhaps because those in good health tend to quit the register, are in this case entirely untenable.

The status of this important contribution to the understanding of AIDS must nevertheless be clearly appreciated. It is well known that no amount of statistical argument can by itself prove that a disease is actually caused by the agent with which it is statistically associated. That is why some people still believe that cigarette smoking is not a cause of lung cancer and cardiovascular disease, for example. Even now, there will be ingenious people constructing hypotheses by which the association described can be explained by mechanisms other than those that appear to stare one in the face.

The view that AIDS is caused not by HIV as such but

by the injection of drugs of various kinds, "recreational drugs" particularly, and also drugs used as medicines, will nevertheless be more difficult to sustain in the light of the evidence from Darby *et al.* The ten-fold increase of the death rate among those carrying HIV will seem to most people to be sufficient, although it entails the assumption that the treatment of patients for their haemophilia would have depended only on the severity of the disease, not on whether they were infected with HIV.

Yet it is safe to predict that there will be complaints from the obstinate community of the unconvinced that Darby *et al.* have failed to provide full details of the drug regimen followed by the 6,000 people on the register, and that until they do, their conclusion has no force.

The mistake in that opinion, if voiced, would be two-fold. First, the data now published are of interest and importance in their own right, as further evidence (if such were needed) that HIV is almost invariably linked with AIDS. As such, they are addressed to the research and medical community at large, not to the company of the unconvinced. Second, the sad truth about debates on controversial issues in science is that there may come a point at which dissenters forfeit the right to make claims on other people's time and trouble by the poverty of their arguments and the exasperation they have caused. The world (to judge from *Nature's* postbag) is full of people who believe that Einstein's relativity is a pack of lies, but who cannot make the claim on other people's attention they would wish.

The tragedy, in the case of HIV and AIDS, is that disbelief in the role of HIV in AIDS has spread from beyond a small company in the research community to a large part of the AIDS community itself. The reasons are unremarkable and pathetic in the strict sense of that word: it is at least uncomfortable for an infected person to know that HIV infection will lead eventually to AIDS. Not for nothing is the knowledge often called a "death sentence". The remedy is not, of course, to pander to wish-fulfilment, but to redouble effort in the laboratory and the clinic. Those who have made the running in the long controversy over HIV in AIDS, Dr Peter Duesberg of Berkeley, California, in particular, have a heavy responsibility that can only be discharged by a public acknowledgement or error, honest or otherwise. And the sooner the better. □



British schools fantasy

The British government should not seek to make its second school-leaving examination even more exacting.

WILL the British educational system (outside Scotland) never be put on a rational basis? This summer, as always, pundits and people more generally have been brooding about the annual crop of results of the school-leaving examinations held earlier in the year. The results come in two forms, for there are two systems of school-leaving examinations. For 16–17 year-olds, there is a system called GCE, for “General Certificate of Education”. Then there is a series of examinations for 18–19-year-olds, called A-levels, where “A” stands for “advanced”. Why two? Because the A-level system has traditionally been aimed not at educational goals, but has been the means by which young people prepare themselves for university courses and also demonstrate their competence to benefit therefrom.

Now a strange thing has happened. Because the numbers of those gaining respectable qualifications at A-level have been rising and have further increased this year, there has been a groundswell of indignation that the examinations have become “easier”, and that educational standards in British schools are falling. While an objective observer might conclude that an increase in the numbers of those qualifying as university entrants would be a sign of *improving* standards, the opposite opinion has paradoxically been strengthened by the discovery that some universities have been recruiting into “foundation courses” young people without A-level qualifications of any kind. Everything is going to the dogs seems to be the view, notably that of the *London Times*.

Mrs Gillian Shephard, an able minister who now looks after both education and employment, seems to have been caught up in the general panic. There is to be an enquiry, on which her department will spend £100,000, to tell whether educational standards are falling and to give new backbone to the A-level system. Sadly, the outcome cannot but entail more short-term administrative tinkering with a system that has long since outlived its usefulness and function. It will not lead to the more radical change in the pattern of secondary education, at the vital interface with higher education, that circumstances require.

The A-level system, once described by a former prime minister as the “jewel in the crown” of British education, has become an impediment to learning rather than a means by which young people in Britain (outside Scotland) can be prepared for adult life. To be sure, in the life of a secondary school, it is enlivening for many teachers and a proportion of the students that they should rehearse together curricula that really belong in higher education, but that requires a high price (in impoverished education) from those who will not end up in higher education. But even academically inclined young people are crippled by the system, being required to abandon general for specialized curricula too soon in their careers.

The rational reform of this bewildering set of circumstances has been plain for many years: require that secondary schools provide a general education for their students, including the cultivation of scholarship that will enable some of them to profit from further study at universities and elsewhere, but also emphatically require that secondary schools do not run a selection system for the universities and other higher education institutes. Over several years, there have been repeated attempts to loosen the present system, mostly by seeking to persuade universities to be less exacting in what they require as specialized knowledge from potential entrants, but with only little success. Yet in a climate in which universities and other institutes are being repeatedly told that their future hangs on the decisions on educational policy they make for themselves, is it not proper that they should be responsible for their choice of students?

It is especially sad that Mrs Shephard has apparently taken seriously the *canard* that some universities (mostly institutions recently graduated from the status of polytechnic) are offering foundation courses to students without qualifications at A-level. At worst, that is means of providing extra education to young people who manifestly both need and seek it. But such a pattern of higher education is also educationally desirable. It is also the pattern followed in most other countries in the world than Britain. Especially when Britain is constantly looking over its shoulder at the performance of its competitors elsewhere, why should it set its face against a pattern of education that appears excellently to have met the needs of, for example, Japan and the United States.

The British pattern is especially disadvantageous for the recruitment of young people into science. Opting for A-level studies of a kind likely to win a place at a university requires a decision to that effect at 16 or 17, and often denies a young person the chance to follow more general aspects of the curriculum on offer at his or her school. That is also an age at which young people take more than passing interest in the prospects that their studies will largely determine their careers, influencing both the chance that they will find a job and that it will reward them well. Yet that is precisely the age at which, to many young people in present circumstances, keeping options open seems the wisest strategy. So long as a career in science requires the almost monastic preparation now customary in Britain, the numbers of entrants to science courses in higher education will continue to dwindle.

Effecting change on this scale would not, of course, be simple. Universities would have to abandon the present practice that young people are mostly recruited not by the university but by its departments. Some teachers would have to teach more elementary classes. There would be a redistribution of costs between schools and higher education (but probably not as great as simple arithmetic suggests). Universities would find themselves standing where they belong, both in respect of accountability for their use of public funds and of academic freedom — on their own two feet. If Mrs Shephard wishes to make a mark with her enquiry, that is the direction she should explore. □

Industry backs 'downsizing' public research, but not joint projects

Washington. Industrial research directors in the United States are backing cuts in government research and development budgets being proposed by the Republican-dominated Congress as giving universities and federal laboratories a much-needed impetus to improve their performance.

At the same time, however, they fear that the closure of technology support programmes will severely inhibit US industry's ability and willingness to carry out high-risk research and development (R&D).

This double message has come from a forum of 60 research bosses from companies such as 3M, DuPont, Mobil and General Motors, organized by the Industrial Research Institute (IRI) and held in Washington late last month to discuss the impact of Congress's budget plans.

According to a summary of the meeting prepared by Chuck Larson, executive director of IRI, the proposed cuts are "a healthy provocation for self-examination and improvement by the federal labs and universities". He points out that industry has itself been 'downsizing' for the past ten years, echoing the response of many industrial research managers to the cuts now facing the publicly-funded research infrastructure. "A little squeezing sometimes helps all of us," says David Knowlen, director of technical affairs at Boeing and co-chair of the forum.

But the forum expressed deep misgivings about Congress's plans to cut back on technology programmes which directly support industrial R&D. These proposals could "turn the clock back 10 or 15 years," the forum summary said — a reference to concern that US government policy would return to its position before a desire actively to encourage technology transfer was first enshrined in legislation in the late 1970s.

Two threatened programmes alone — the commerce department's Advanced Technology Program (ATP) and the defence department's Technology Reinvestment Program (TRP) — are now matched by private sector commitments totalling \$5 billion over five years. This is little compared to US industry's total annual R&D expenditure of around \$100 billion. But Larson says that the two programmes may represent up to

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US Department of Energy/SPL

Plugging in? Supporters of the electric car say that federal funds provide fuel for high-risk ventures.

half industry's spending on high-risk R&D, and have created "a 50-100 per cent increase" in what would otherwise be done in this field.

"High-risk R&D is very hard to sell to any board of directors," says Knowlen. "Much of it is supported by partnerships between government and industry." Since the end of the cold war, the Boeing director adds, the amount of research supported for military purposes "has greatly narrowed".

Both ATP and TRP have been targeted for elimination by the House of Representatives. The Clinton administration, however, has promised to defend the programmes, and the Senate is likely to try to preserve at least part of TRP.

Republicans on Capitol Hill claim that industry has done little to defend these programmes, preferring to use its formidable lobbying power to push for deregulation or tax breaks, making it easier to raise capital.

But Knowlen replies that the aerospace industry, at least, has made its position quite clear. The chief executive officers of the seven largest companies wrote to Congressional leaders in May, warning that ending the technology programmes would damage US competitiveness.

The IRI forum revealed some divisions between participants. Jean Bonney, for example, director of external research at DEC and the forum's other co-chair, threw some doubt on the general effectiveness of the ATP and TRP by comparing them to the European Union's much older and larger Framework research programme.

Bonney acknowledged that Framework has encouraged European companies to collaborate. But "if you look at exactly what [Europe] has got, in technology and product terms, we haven't seen a whole lot," she said.

The research directors expressed concern that the US government appears to have been backing away from Co-operative Research and Development Agreements (CRADAs) in the past few months — a trend sensed, for example, by the largest single CRADA participant, General Motors.

The directors also said industry would boost support for university research if the government cuts it, but not by enough to fill the hole this would leave.

Industry could live with reduced graduate output from the universities, they said, given the current record level of supply. Its ability to innovate would be hit by cuts in engineering research, they said, but not in some scientific disciplines — although they were not specific on what these might be.

The forum did not favour the restoration of the Research Tax Credit, a claimed incentive for companies to boost their reported research spending each year that has long been pursued by corporate lobbyists in Washington. It branded the tax credit as "more of a financial tool than a technology tool for most companies".

The House and Senate budget proposals will be reconciled this month, and the fate of the technology programmes sealed in October, when they collide with the Clinton administration's own priorities in a festival of brinkmanship, known as the 'train-wreck', from which final budget numbers will emerge.

Colin Macilwain

Gupta associates feel the reflected heat

New Delhi. The Panjab University in Chandigarh has set up a committee of four faculty members to decide whether, and if so how, to punish two research associates of the geologist Viswa Jit Gupta, who was found guilty last year of faking geological data (see *Nature* 371, 368; 1994).

One of the scientists, Arun Ahluwalia, claims that any such punishment would be unjustified. He points out that a commission of inquiry set up to review Gupta's work established that he and his colleague were victims of Gupta's deception, and alleges that they are now being victimized by the university.

But the university's vice-chancellor, T. N.

Kapur, says that although the commission showed the two researchers to be innocent, consideration now needs to be given to withdrawing any benefits that they may have received as a result of their association with Gupta — as the commission had requested.

The Society for Scientific Values, a group of Indian scientists committed to upholding good scientific conduct, is to consider Panjab University's actions at its October meeting. Meanwhile, according to Avtar Singh Paintel, a former president of the group, the society is writing to the university asking it to stop "harassing" the two researchers.

K. S. Jayaraman

US report raises fears over nitrate levels in water

Washington. The concentration of nitrates in drinking water in agricultural areas of the United States often exceeds safety standards and is increasing steadily, according to an extensive study carried out by the United States Geological Survey (USGS).

The study made use of 34,000 water samples, and is described by the USGS as the most extensive ever undertaken in the United States. It found that 9 per cent of all domestic water wells, and 21 per cent of samples taken from shallow wells beneath farm land, exceeded the nationally accepted level of 10 mg of nitrates per litre of water. Previous, smaller studies had found only 2.4 per cent of wells exceeded that level.

High levels of nitrates have not been proved to be a hazard for adults. But they have long been regarded as dangerous for infants under six months of age, in which they can cause methaemoglobinaemia, or blue-baby syndrome. The Environmental Protection Agency recommends that parents who use domestic wells test for nitrates, and use bottled water if levels are high.

The survey found that only 1 per cent of public water supplies contained nitrates in excess of the accepted level. But far higher levels were found in domestic wells, on which millions of US households depend.

The study is published at a critical time. Congress is considering revisions to the Safe Drinking Water and Clean Water acts which, environmentalists say, would remove restraints on the use of the bulk fertilizers which, they claim, are the dominant factor behind growing nitrate concentrations.

Environmentalists are already citing the USGS study as evidence that nitrates are a mounting problem in need of a national response. "The findings of this study are most disturbing," says Brian Cohen of the Environmental Working Group, a Washington-based pressure group. "It indicates we have a real national health problem".

But Ron Phillips, a spokesman for the Fertiliser Institute in Washington, says fertilizer use has been "stable" for the past fifteen years. "There are specific areas of the country where the incidence of nitrates is a concern — but the direct link to fertilizer use is very tenuous," he says. Any attempt to solve the problem nationally would be "impractical and overly expensive".

The survey looked at data from 1970 to 1992, and found steadily increasing levels of nitrates in almost all wells where comparable data were available throughout the period. It said the problem was most severe in largely homogenous agricultural areas, such as the mid-west, and less so in areas such as the south-east, where trees were plentiful and land-use varied.

Colin Macilwain

San Diego research body put under microscope on costs

San Diego. Officials at the University of California at San Diego (UCSD) are investigating how a private foundation partially controlled by faculty members from the School of Medicine spends an annual \$7.2 million that it receives for research and education.

The inquiry has been going on for almost a year because the foundation has refused to open its books to university auditors. The latter have been checking such foundations at other campuses of the University of California after improprieties were found last year at organizations in San Francisco. They are particularly concerned that attempts may be made to use such foundations to circumvent the university's rules on applying overhead charges to external grants.

Officials of the Medical Education Research Foundation (MERF) in San Diego say that its affairs have been conducted properly, and that, as an independent body, it should be able to protect the privacy of donors of gifts and grants, some of which come from pharmaceutical companies.

Henry Wheeler, a retired head of gastroenterology at UCSD and MERF's president for several years, declined to comment directly, referring inquiries to the foundation's attorney, R. Michael Scarano.

Scarano says MERF has "made substantial contributions" to the university campus and the region's medical community, and wants to continue that role. He adds that the foundation is in "amicable discussions" with the university to resolve the dispute.

Current and former faculty members from the Department of Medicine at the UCSD School of Medicine serve on the board of directors of MERF, which was formed in 1976 and is the largest such foundation at UCSD, with \$16.7 million in assets.

University officials say they want to examine financial records to ensure there is no abuse of funds, conflicts of interest or improprieties that could reflect badly on the University of California, in whose name some clinical drug trials are conducted.

The auditors are trying to determine

whether UCSD faculty members used university facilities in any of the 25 to 30 clinical trials conducted annually by MERF, whether MERF paid UCSD faculty or staff for work performed on university time, and whether MERF has retained funds intended for UCSD.

John Woods, a vice-chancellor at UCSD, said all other incorporated foundations and support groups at the medical school have cooperated with university auditors. "We are trying to convince (MERF) to turn over information on their operations," he added.

Last year, California's Bureau of State Audits, a watchdog agency, persuaded the San Francisco District Attorney's Office to conduct a criminal investigation to secure the records of two foundations at the University of California at San Francisco (UCSF), where irregularities were alleged.

No criminal charges were filed. But the auditor's subsequent report found conflicts of interest among UCSF employees, payroll records being falsified, university facilities being used for the benefit of personnel, and imprudent spending on gifts and entertainment. The state Attorney General's Office is continuing to investigate the UCSF case.

Ward Connerly, chairman of the finance committee of the University of California Board of Regents, says that he is concerned that foundations like MERF have used their organizations to avoid paying university overhead costs for research. He claims that the foundations "did this to consciously get around university policies".

But MERF attorney Scarano denies such charges. He says that the foundation was trying to "streamline" the grant handling process so that "UCSD faculty would not lose the opportunity" to participate in the clinical drug trials.

According to UCSD officials, an overhead rate of 51 per cent is currently charged on federal grants to the university. UCSD is currently implementing a new policy for grants received from foundations, with a special overhead rate of 19.5 per cent.

Rex Dalton

France still follows in Verne's footsteps

Paris. The Francophone world, already one of the largest consumers of popular science magazines, will next month have another title on its shelves with the launch of *Eurêka* by Paris-based publisher Bayard Presse.

The monthly 84-page magazine will cover all areas of science, and sell for FF19, about US\$4.00. Bayard has set itself a circulation target of 150,000.

Eurêka marks the return of Bayard Presse to popular science publishing after a

50-year break. In 1852, the company launched *Cosmos*, one of the earliest popular science magazines in France. *Cosmos*, which counted Jules Verne among its subscribers, was last published in 1940.

A survey carried out by Bayard in association with the pollster SOFRES in June found that 30 million French — about half the population — claim to be interested in science, and that 6 million read science magazines.

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Biologists challenge museum shake-up plan

Cape Town. A task force set up by South Africa's minister for arts, culture, science and technology, Ben Ngubane, has set off a furore among biologists with a proposal that the country should have two national museums, one in each of its existing capital cities of Cape Town and Pretoria.

According to the proposal, made by the Arts and Culture Task Group (ACTAG), the two institutions will bring together ten of the present eighteen national museums. But only two of these are natural history museums: the South African Museum in Cape Town, the largest in the country, and the Transvaal Museum in Pretoria, which has the biggest collections.

As a result, the remaining three national museums with natural history collections — the Natal Museum, the National Museum in Bloemfontein, and the Smith Institute of Ichthyology in Grahamstown — each stand to lose their national status and fall under provincial control.

The ACTAG recommendation has provoked substantial criticism, not least because none of the seven members of the task force's heritage subcommittee, responsible for providing guidance on museum policy, is a biologist, or has any experience of working in a natural history museum.

Ironically, the Smith Institute's collection of three-quarters of a million fish specimens makes it the only one of the five natural history museums which is truly national. The other four still reflect to varying degrees the regional bias of the former colonies in which they were set up, and which subsequently became provinces of South Africa under the old constitution.

Naas Rautenbach, Director of the Transvaal Museum, points out that the two new national museums being proposed are in the country's richest provinces, the Western Cape and Gauteng. The likely fate of museums in the remaining seven provinces is difficult to predict, he says, given other provincial priorities. Political leaders in some "appear to lack an understanding of the value of research and collections, although they do seem to appreciate the educational value of museums", he says.

Rautenbach adds that the decision does not augur well for South Africa's ability to fulfil a commitment made in the Rio Declaration of 1992 to draw up a complete inventory of the country's biodiversity before the end of the decade.

"Instead of going forward with this task, we run the risk of going backwards," he warns. Museums, as acknowledged repositories of specimens with known localities, are

the major source of scientific information for most animal groups.

The task group's recommendation also assumes that the country will decide to maintain its existing dual capitals. But last month Patrick Lekota, premier of the Free State, threw a spanner in the works by offi-



Cape Town's South African Museum: soon to be one of a lucky pair?

cially proposing that Bloemfontein, which is in the centre of the country, should become the national capital.

ACTAG rejected a suggestion that four national museums be formed out of all the existing eighteen museums which are currently national institutions. These would become museums of arts, culture, science and technology (including natural history) and military history respectively, each made up of a group of museums in cities around

the country.

Even given the problems of long-distance administration, many see this as a more workable solution. Furthermore, this would make it possible for the natural history museums to rationalize their collections either on a taxonomic or a regional basis.

The difficulty with both proposals is that they exclude several excellent museums, currently run by municipal or provincial authorities, from any rationalization at the national level. Yet the Durban Museum of Natural Science — the country's most popular museum — as well as the Port Elizabeth,

Albany, East London, Kaffrarian and McGregor Museums, all have natural history collections of national value.

ACTAG appears to have completely ignored a third alternative, namely that all natural history museums be linked to the provinces in which they are located — as in Australia. Their financial future could be secured through earmarked funds provided to the provinces by the national government.

Michael Cherry

Butterfly 'smugglers' take to the wing

New Delhi. India is seeking the return of two German nationals who were caught at New Delhi airport last year while trying to smuggle 14,000 butterflies and moths out of the country. The Germans are being sought for trial under a domestic law which makes the smuggling of protected species a punishable offence.

The pair were stopped by customs officials on 14 August 1994. They had travelled to India as tourists, and had captured the insects from the Himalayas using local help — but without official permission.

Not all butterflies are protected in India. But the government's wildlife department had wanted to detain the Germans until all the insects in their collection were identified, in order to discover if any of them belonged to a special protected list.

But species identification is a time-consuming exercise. And the government — anxious to avoid a diplomatic row — agreed to let the two Germans leave the country after their embassy in New Delhi had given a written guarantee that they would be brought back to India to stand trial if, at the end of species identification, they were found guilty.

The Calcutta-based Zoological Survey of India has now made detailed identification of 400 butterflies and 2,000 moths from the

tourists' collection and found that they included four species from the protected list. Wildlife officials say that the pair have clearly violated the Wildlife Protection Act (1972), and should be put on trial.

A spokesman for the German embassy in New Delhi confirmed that it has received a note from the Indian Ministry of External Affairs, accusing the two tourists of violating the wildlife act and seeking their return to stand trial. He said that his embassy has asked the Indian government to provide it with "more details of the charges, in order to inform the concerned authorities back in Germany".

Friends of Butterflies, a Delhi-based non-governmental organization, is worried that the delay in taking both the two German tourists and their local contacts to court may send the wrong signals to organizations that are engaged in the illegal multi-million dollar butterfly trade.

Virender Singh, the organization's coordinator, said that rare species of butterflies have been disappearing from the Himalayan states, as well as from the Andaman and the Nicobar islands in the Bay of Bengal. Many are ending up in so-called 'butterfly houses', making money through entrance fees charged to visitors to museums.

K. S. Jayaraman

Can OSPREY rise up from its watery grave?

London. The developers of OSPREY, the world's first commercial wave power-station, appear unfazed by its sinking off the Scottish coast in storm conditions less than a month after its launch on 2 August.

They have pledged to build its successor, OSPREY 2, by next summer — despite renewed doubts about the device's ability to withstand the region's harsh weather.

OSPREY stands for Ocean Swell Powered Renewable Energy. Funded by private companies and a £435,000 (US\$700,000) grant from the European Union's Joule programme for research into non-nuclear energy, it was designed to generate electricity for 2,000 homes for 25 years.

As such, OSPREY was billed by its supporters as a sharp riposte to those — including the British government — who have dismissed wave energy as unsound, uneconomic and, therefore, unworthy of state support (see *Nature* 376, 544; 1995).

But less than two weeks after its launch, continued buffeting by waves had led to holes in OSPREY's ballast tanks. The structure then caught the tail end of Hurricane Felix, and by 26 August it began to sink.

Despite this, Applied Research and Technology (ART), the Inverness-based engineering company that designed and funded the £2-million generator, described the incident as just an unfortunate setback.

Allan Thomson, the company's managing director, says he intends to push ahead with the project which, he says, should be completed "in the next six months". He expects it to be funded out of the insurance money received for the sunken OSPREY. "Nothing will change," says Thomson. "All our backers are still with us. The market interest from maritime countries continues to grow. There will always be hurdles, but you just have to cross them."

Thomson refuses to speculate on why OSPREY sank. The generator was insured with Lloyds, and an assessor's report is expected shortly. But Thomson strongly denies that the sinking was the result of any flaw in OSPREY's design, which he says will remain unchanged for OSPREY 2. "The design was good. The installation went like clockwork. We are not likely to change the design. But we will speed up the installation [for OSPREY 2]."

Prior to launch, critics had also questioned the decision to launch the OSPREY off Scotland's north coast. Donald McDonald, an electrical engineer at Imperial College, London, was reported as saying that the weather was sufficiently rough in winter that only granite lighthouses stood any chance of staying upright. But Thomson says

that the coast off northern Scotland has excellent wave conditions. "It is the perfect location. I have absolutely no regrets".

All of OSPREY's private backers — including British Steel, GEC Alsthom and Scottish Hydro-Electric — remain firmly committed to the scheme. But the position of officials responsible for the EU's Joule programme, which recently awarded

ECU800,000 (US\$1.05 million) to the University of Edinburgh to develop a

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

That sinking feeling: the power generator after last month's launch (above) and shortly before disappearing under the waves off Scotland's northern coast (right).

hub for a wave generator's two-tonne turbine blade, remains unclear.

Wolfgang Palz, of the renewable energy division of the European Commission's Science, Technology and Research directorate, says any future decision to fund an OSPREY project will take the sinking incident into account. "That does not mean the answer will be an immediate, 'no,'" he emphasizes. "Our technical experts will take a look, make an assessment and draw the appropriate conclusions."

But Palz also maintains that OSPREY's fate will not affect the Joule programme's support for the concept of wave energy. "Wave energy should have its chance; it is at an early stage and has huge potential" he

says. "Accidents happen, as we have to deal with a hostile environment. It is normal for things not to go as much to plan as they do for solar projects, for example."

Wave energy research in the United Kingdom has been largely privately funded since 1983, when the government pulled the plug having spent nearly £17 million over 10 years unsuccessfully trying out 300 different concepts of harnessing energy from waves. Between 1985 and 1993, the government spent just £2 million, mainly to support work on a 75kW oscillating water column device developed by the Queens University of Belfast.

The Write Image

Researchers at the University of Edinburgh, where much of Britain's wave energy research has been carried out, accused the government of withdrawing at a critical period when a breakthrough looked increasingly likely.

But a report on wave energy published in 1992 by the Department of

Trade and Industry, which is now responsible for UK energy policy, concluded that the main devices developed were unlikely to generate electricity competitively in the short to medium term.

The review recommended that the freeze on funding for wave studies remain, on the grounds that "the technical performance and reliability of many components over a long term in a marine environment should be demonstrated".

A report produced earlier this year as part of the government Technology Foresight exercise reached a similar conclusion, recommending merely that a "watching brief" be kept on both wave and tidal energy sources.

Ehsan Masood

Greenpeace under fire on Brent Spar coverage

London. Television news editors last week admitted that they had been manipulated by the environmentalist group Greenpeace over the coverage of the Shell Oil Company's abandoned plans to dump the Brent Spar oil platform in the North Sea. This was partly, they say, because "seductive" pictures make better television than good science.

"Greenpeace is the best and most professional pressure group," said Richard Sambrook, a senior BBC news editor, during an address to last month's Edinburgh television festival. "It can target more resources at one story than a news organization can, and provide better, more compelling, more frequent coverage," he admitted.

Sambrook said that the BBC tried to redress this by broadcasting reports

analysing scientific arguments, explaining the context, trying to give the bigger picture. "But such attempts at retaining our editorial propriety are not as memorable as seductive pictures from the North Sea."

He added that reporting issues such as the proposed dumping of the Brent Spar was made more difficult for news editors by the conflicting scientific claims of either side, including the fact that "even independent scientists" find some of these issues difficult to agree upon. "We are lay men and women caught up in the crossfire of a battle of statistics."

But in a newspaper article, Chris Rose, programme director of Greenpeace UK, claims that Sambrook's comments are "patently untrue". □

Rule change curbs grants to European labs

Munich. European-level laboratories may no longer be able to receive grants from the European Commission (EC) for scientists from European Union (EU) member states to use their large-scale facilities, under a new interpretation of the rules governing the allocation of EU research funds.

This new interpretation, which is intended to focus support on EU scientists using national facilities in another state, may have wider implications, as the commission is discussing whether it should also apply to all guest scientists wishing to work at any European-level laboratory.

The European Molecular Biology Laboratory (EMBL), which has its main laboratory in Heidelberg and two outstations at synchrotron facilities in Grenoble and Hamburg, is the first to be affected by the apparent change in the commission's philosophy.

EMBL has been informed unofficially that grants received under the Human Capital and Mobility programme of the EU's third Framework programme, which ran from 1990 to 1994, will not be awarded under the equivalent programme in the fourth Framework, called Training and Mobility of Researchers (TRM).

The laboratory had received nearly ECU3 million (US\$3.85 million) from the third Framework to improve research equipment for visitors using neutron beams at its outstations, and to equip a supercomputer centre associated with its main laboratory.

The funds were also used for a major visitor programme for EU scientists. "If the

money is not renewed, it will be a great loss," says the Grenoble outstation director, Steve Cusack.

The basic philosophy of the new TRM programme has not changed from that of its predecessor, namely to promote the movement of researchers between EU countries. But some details have changed in the way that funds are used. Equipment costs, for example, are not being financed this time. And all applications will be considered by a single multidisciplinary committee, rather than by a series of scientific assessment committees divided along disciplinary lines.

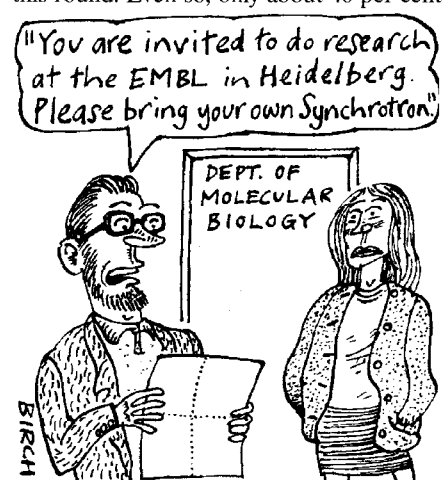
The members of this new committee, which met in July to make its recommendations for the first round of the TRM Large Scale Facilities programme — the part of the overall programme designed to support work at large research facilities — have been told that they should seek to maximize the access of European scientists to European facilities in general, at both national and European levels, as well as judge applications on their scientific merit.

According to Allan Mackintosh, the committee's chairman who is a solid-state physicist from the University of Copenhagen, since most EU member states are members of EMBL (only Portugal and Luxembourg are not), they already have access to its outstations. As a result, EMBL's bid for support was not judged to be a cost-effective way of using the TRM budget.

"It is not true to say that European-level laboratories are not eligible," says Mackin-

tosh. "It's just that they couldn't compete on this criterion [of cost-effectiveness]."

The Large Scale Facilities programme has been allocated a total of ECU110 million in the fourth Framework programme, of which ECU72 million is being distributed in this round. Even so, only about 40 per cent



of the 200-odd applications received, whose general standard was high, could be funded, says Mackintosh. A second call for proposals will be made in 1997, prior to the distribution of the remaining funds.

The commission is also very unhappy about frequent leaks from TRM programme committees, despite the fact that their members are required to pledge confidentiality. This time the source of information leaked to EMBL has been identified, and a commission spokesman says that the individual concerned will not be asked to sit on any future EC committees.

A formal confirmation by the commission of the TRM Large Scale Programme committee's recommendations is due later this month. "If a formal decision is taken that the money will not be available in future, we will have to move cash from elsewhere [in EMBL's budget]," says Barton Dodd, the laboratory's administrative director. "It will have serious implications for us."

EMBL is already experiencing difficulty in raising funds that it is seeking from its member states to support an expanded programme in the period 1996–2000, including growth in its visitor programme.

The commission's new interpretation of the TRM programme rules may have even wider implications. According to the German delegation, the TRM programme committee, which is made up of two delegates from each EU member state, is considering whether to disallow, or at least 'deprioritize', bids for funding from the human mobility section of the TRM programme for scientists wishing to work at EMBL and other European facilities. This issue will be discussed by the committee in December.

Alison Abbott

Chinese agree to eugenics discussion

London. The International Genetics Federation (IGF) has withdrawn a threat to abandon its choice of the Chinese capital Beijing for its next five-yearly Congress, following an agreement by the Chinese organizing committee that the meeting will include a symposium on eugenics.

The IGF was considering abandoning Beijing for the 1998 International Genetics Congress in protest at the Chinese government's decision to introduce a new 'eugenic' law designed to reduce 'inferior births' by forbidding people with inherited diseases from marrying (see *Nature* 372, 123; 1994).

The law, which came into effect on 1 June this year, is a watered-down version of a bill that was heavily criticized by the international scientific community when proposed in January 1994. Members of the IGF's executive committee had voted in March to find an alternative venue unless their Chinese hosts agreed to hold "a full discussion of eugenics" during the 1998 congress.

In a statement issued last week, A. J. F. Griffiths, secretary to the IGF, said this

request had now been met and the congress would therefore proceed as planned. The eugenics session, he added, will cover a range of scientific and ethical topics. "It is expected that speakers will include experts in various areas such as history, ethics, population genetics and medical genetics."

David Smith, of the University of Birmingham and president of the IGF, says convincing the Chinese organizers to set aside time for the symposium had not been easy. A key issue for the Chinese scientists had been their concern that the symposium might be used as an occasion for attacks on the government.

But Smith adds that the Chinese authorities have promised not to interfere in the running of the programme, or to stop any delegate from attending on political grounds. The genetics congress, which was first held in London in 1899, has had to change its advertised venue just once in the past. The 1939 congress opened in Edinburgh, Scotland a few days before war broke out against the original host, Nazi Germany.

Ehsan Masood

Republicans challenge 'misuse' of scientific data by US agencies

Washington. A series of Congressional hearings opens next week, designed to investigate Republican allegations of the misuse of science by the Environmental Protection Agency (EPA) — as well as by other branches of the federal government — to justify new environmental regulations.

The hearings are being held by the energy and environment subcommittee of the House science committee. They will explore various politically-sensitive scientific issues, such as the carcinogenic effects of dioxins, the role of chlorofluorocarbons (CFCs) in depleting the ozone layer, and the dangers of global warming.

Dana Rohrabacher (Republican, California), the highly conservative chair of the subcommittee, plans to use the hearings to seek evidence that the US government has misused scientific data to 'prove' threats from dioxins, CFCs and greenhouse gases, in order to justify regulations that others consider unnecessary. Prominent scientists who dispute the scientific consensus on each of these issues will be called as witnesses.

The first hearing is scheduled for 13 September, and will examine how the EPA carried out earlier this year an important reassessment of the risk of contracting cancer from exposure to dioxins. Rohrabacher's staff are particularly concerned about how the EPA completed the reassessment.

According to Harlan Watson, staff director of the subcommittee, the eight chapters in the body of the report were properly peer-reviewed. But the ninth, summarizing the report, was "written in-house by EPA-types, and not properly peer-reviewed".

A week later, on 20 September, Rohrabacher's subcommittee plans a hearing on the accelerated phase-out of CFCs, the solvents which were banned after scientists had established their role in depleting the ozone layer in the upper atmosphere. Witnesses include what Watson calls "some pretty sceptical folks" — including scientists who still dispute the need for the ban.

No date has been scheduled for the third hearing in the series, which will question the need for international agreements on the stabilization of greenhouse gas emissions. Rohrabacher has previously described global warming as "liberal claptrap", and is expected to track down witnesses who share his view.

Colin Macilwain



Rohrabacher: concern at dioxin conclusions.

Internet Expo provides boost to Asia's computer networks

Tokyo. Asian governments are leaping eagerly at the chance to participate in next year's Internet World Exposition in Seoul, the capital of South Korea. Many see the exposition as a golden opportunity to persuade their citizens of the importance of computer-based communication.

Last week South Korea's Minister for Information and Communications, Kyong Sang-Hyon, announced his country's official support for the fair. Japan's preparations are already well advanced, China, Singapore and Thailand have recently announced they plan to participate, and other countries in the region, including Malaysia, are expected to follow suit.

The exposition, the brainchild of cyberspace pioneers such as Vinton Cerf, will run from January to December 1996, and is an attempt to emulate past world fairs, which many see as starting with the 1851 Great Exhibition at the Crystal Palace in London.

One of the achievements of such fairs has been to introduce millions of people to the latest marvels of science and technology, such as electric light and power. The Internet Expo is similarly intended to demonstrate the enormous benefits of computer connectivity, and has already won the support of the Clinton administration.

Asia currently lags far behind the United States and Europe in terms of network infrastructure and number of on-line subscribers. "Just for Japan, the expo will increase connectivity by ten times," predicts Carl Malamud, one of the fair's principal organizers, who has been travelling around Asia, signing up participants.

South Korea has designated its National Computerization Agency as the secretariat for the expo. Japan has yet to issue a formal statement of support. But in terms of fundraising and corporate backing, Japanese efforts are currently the most enthusiastic.

International telecommunications carrier KDD, for example, has agreed to donate a high-speed T3 optical fibre link, capable of transmitting 45 megabits a second, for trans-Pacific connection to the United States, for

the duration of the expo. At the same time NTT, Japan's largest domestic carrier, will provide similar connections to 15 sites — 10 cities and 5 universities — in Japan.

To encourage the participation of individual users, NTT will also set up free digital links to 300 Japanese homes. These will run at 128 kilobits per second, the fastest speed that conventional copper cables can handle.

Manufacturing companies such as Sony, as well as leading Japanese software houses like ASCII and Softbank, have pledged cash, equipment and manpower worth US\$10 million. A meeting of the Japanese expo working committee last week in Tokyo attracted almost 100 participants. The committee is chaired by Jun Murai, an associate professor at Keio University and the *de facto* leader of the Internet community in Japan.

Last month, Murai went to China to stimulate support for the fair. "Chinese universities are very interested in working with us," he says. Murai returned from Beijing with a memorandum of understanding signed by the vice president of the Chinese Academy of Sciences, Lu Xiankui, stating that China will participate.

As China lacks high-speed optical fibre links, Murai has arranged to borrow a transponder on JCSAT-II, a new satellite launched by Japan on 29 August. The transponder's 18 circuits, each operating at a speed of 2 megabits per second, will also provide connections with India and other parts of Asia.

A high-speed link connecting Japan and South Korea is also under discussion. All these connections could well constitute what Asia has lacked so far, namely a transcontinental Internet 'backbone'. Asian countries currently hook up to the Internet via separate links to the United States.

The expo will last for just a year. But it is likely to be of lasting significance for Asia. Malamud points out that the high-speed backbone network set up by the National Science Foundation was only temporary, but the commercial Internet developed out of it.

Bob Johnstone

Europe agrees closer research links with Israel

Paris. The council of ministers of the European Union (EU) and the government of Israel are scheduled to sign a trade agreement next month, which will allow Israel to take part in the EU's Framework research programmes.

Agreement had been expected last spring, but had been delayed because of wider negotiations over trade issues (see *Nature* 375, 723; 1995). Under a draft agreement whose contents were approved

during the summer, Israel also won a concession from the EU allowing it to sit on the committees that administer research programmes, even though Israel will not have voting rights on these committees.

Following next month's signing of the trade agreement — which Israel hopes will help reduce its \$8 billion trade deficit with the EU — the text will need to be approved by the European Parliament and ratified by the parliaments of all EU member states. □

Patent row splits fish identity researchers

Paris. A bitter conflict has arisen over a patent on a technique for tracing the genetic origin of canned tuna fish, stirred by overlapping disputes over the validity of the patent itself, and its implications for the relationship between rival research teams.

The conflict is pitting taxonomists and other researchers, backed by the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF), against the holder of the patent, the Canadian company Bio-ID, and its European licensee, Atlangène of France.

The patent, which describes a technique for identifying the tuna species from which a sample has been taken, has been granted in both the United States and Europe. The so-called FINS (Forensically Informative Nucleotide Sequencing) technique involves isolating DNA from the sample, amplifying it with the polymerase chain reaction, and comparing its nucleotide sequences with those of known species in a database.

The patent was granted to William Davidson and Sylvia Bartlett of Memorial University in Newfoundland. Bartlett and Davidson — the latter associate dean of science at the university — later transferred their rights to Bio-ID, a company set up jointly with the university, and of which they are respectively president and vice-president for research and development.

The two scientists originally developed the technique for Canada's Department of Fisheries and Oceans. The department wanted to identify carcasses of blue-fin tuna in order to prevent poaching of the regulated species, which can fetch Can\$1,000-20,000 in Japan's sashimi market. Davidson claims that the development of FINS has already stopped such poaching.

Last month, however, Susan Pryde, a researcher at MAFF's Torrey Marine Research Station near Aberdeen in Scotland, launched an appeal to researchers via the Internet for papers demonstrating that the patented technique was not novel. Pryde says Atlangène has told her that her own work on the identification of tuna species infringes the Bio-ID patent.

MAFF says it does not believe the Torrey group's research infringes the patent, as it is "not using the test commercially" — under European patent law scientists are allowed to use a patented process for research purposes without needing either a licence or the permission of the patent holder.

But the outcome of similar patent conflicts in the past suggests that Atlangène may have a case. The MAFF group's research appears to be aimed at developing the technique patented by the Canadians, and the dispensation given to research under European patent law does not apply to research carried out to satisfy a regulatory body.

Indeed, Pryde admits that MAFF may be interested in eventually using the technique

for enforcing regulations, in which case it could seek a "crown licence": a compulsory licence of a patented technique which can be obtained when a government wants to use it to enforce legislation.

Although Pryde argues that "we wouldn't want to commercially exploit a test as such, but only use it to protect consumers", jurisprudence again suggests that research aimed at applying for compulsory licences counts as commercial use under European patent law (see *Nature* 376, 622; 1995).

Both Davidson and Atlangène argue that the Torrey group's position seems self-contradictory. "If they aren't wanting to commercialize the work, why do they want to challenge the patent?" asks Davidson.

Much of the tension between the warring parties seems to stem from a long-running dispute between the Torrey Laboratory and the patent holders. In a letter sent to the European Commission in October 1994, Davidson and Bartlett challenged a grant application by the Torrey laboratory on the grounds that the project would duplicate the efforts that have already been made to develop techniques that are now in commercial use in Europe and North America.

"You can imagine our surprise when we learned that the European Community was awarding a substantial grant to a consortium from the UK, Germany and Spain" to develop exactly the same process, says Davidson, who suggested last year that, if the commis-

sion wanted to award such a grant, it would be "more appropriate" to award it to Atlangène.

Pryde claims that her attempts to stimulate a challenge to the patent are based on the grounds that the technique it describes are "not novel". She also predicts that, having negotiated rights to the Bio-ID patent, Atlangène "is not going to leave us, or others using similar techniques, alone."

But the Torrey group is also aware that a successful challenge to the patent would put an end to any infringement claims by Atlangène. MAFF officials say that they are "keen to see the patent challenged", given, it claims, that it was based on research "that was already in the public domain." Indeed, MAFF itself is believed to be preparing a

formal challenge to the patent. Davidson describes Pryde's Internet appeal as disingenuous, arguing that while Bio-ID may contest applied research such as that carried out at Torrey, it has no intention of blocking fundamental academic research. "We don't, nor shouldn't, expect royalties or other fees if the process is used strictly for academic research", he says. "We're completely happy about that sort of use."

Atlangène says similarly that the patented identification technique can be freely used by any laboratory wishing to use it for non-commercial purposes such as fundamental research in phylogenetics.

But Pryde's Internet appeal has struck a nerve among taxonomists and researchers in molecular evolution worldwide, who fear the patent could still impede their research. One scientist who attended a workshop on molecular evolution held at Wood's Hole in Massachusetts last month said that participants were "astonished" that such a patent had been granted.

One reason is that many researchers feel that the patent should not have been granted because of the extent to which FINS is based on techniques which were widely used when Bartlett and Davidson filed their application in 1990.

They disagree with the conclusion of the European Patent Office that research papers describing similar techniques, which were published before the patent application, did not constitute "prior art", and have been trawling the literature for other papers that could be used in any challenge.

Sir Alec Jeffreys, for example, the British researcher who pioneered (and successfully patented) some of the original techniques for DNA 'fingerprinting', said this week he did not see any novel products in the work that would be appropriate for patenting. "I would be extremely alarmed if any patent agent granted this patent," says Jeffreys.

Bio-ID and Atlangène are both refusing to comment on statements by Pryde and others statements about the validity of the patent in the absence of a formal challenge. Davidson admits the patented procedure is based on established techniques. But he points out that the patent offices on both sides of the Atlantic have accepted their claim that "the combination of such techniques made [the patent] inventive".

Some researchers are also concerned about the breadth of the Canadian patent, which covers not just tuna fish but all "eukaryotes and prokaryotes". Davidson maintains that Bio-ID would press for infringement not just for cases involving tuna fish, but also for cases covering tigers to bacteria.

Declan Butler

IMAGE
UNAMBIGUOUS
FOR COPYRIGHT
REASONS
REASONS

Murky waters: a blue-fin tuna, at the centre of dispute over the patent on a genetic identification technique.

Planet Earth Pictures

Eradicating nuclear weapons

SIR — In your apology for nuclear proliferation (*Nature* 376, 371–372; 1995) you say that the “taste of the forbidden fruit cannot be forgotten”, but the rest of the leading article fails to do justice to the memory of Hiroshima and Nagasaki. It is the opponents of nuclear energy and weaponry who are trying to preserve the memory of this “fruit” while the nuclear powers are doing their best to forget. You welcome the Comprehensive Test-Ban Treaty as inhibiting the development of new weapons, but this view is contradicted by France’s declaration that it will stop testing only when it has perfected the capability of simulating explosions by other means. France, and presumably the other nuclear powers, will then retain the means for developing more weapons. Unless the nuclear powers make an unambiguous commitment to disarm there can be no incentive for other nations to respect the Non-Proliferation Treaty.

It is often asserted that the two bombs dropped on Japan were necessary to complete victory over fascism. But it appears that fascism won the more lasting victory by establishing mass annihilation of civilian populations as a valid strategy of war. The West has spoken many fine words about human rights, but actions speak louder than words and few actions speak louder than these two bombs. It may be that nuclear weapons bring short-term benefits for their owners (although your statement that there has been “no armed conflict of any kind in Western Europe” can only be justified if you regard Northern Ireland as part of another continent), but there is absolutely no evidence that they have made the world peaceful in the past 50 years.

There are obviously enormous difficulties in the way of complete eradication, but since we have completed the by no means insignificant task of splitting the atom, it would be irresponsible to abandon attempts to control the consequences. As you say, the dangers of nuclear war are well known. There is a real possibility that international cooperation based on this common knowledge would develop if only the nuclear powers could be persuaded to give up their weapons of mass destruction.

Martin Juckes

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SIR — In a leading article “Physics not guilty”¹, you say that “Oppenheimer had earlier argued against the bombing of Japan, urging a demonstration of the power of the bomb instead”. I think he took the opposite view. Oppenheimer was one of four members of the Scientific Panel (the others were Arthur Compton, Enrico Fermi and Ernest Lawrence) nominated by the Interim Committee appointed in May

1945 by Henry Stimson, the US Secretary of War. In its recommendation of 16 June 1945, the panel concluded that “we can propose no technical demonstration likely to bring an end to the war, we see no acceptable alternative to direct military use”².

You also say that “Oppenheimer’s plea for openness was vindicated, a few months after Hiroshima, by the publication of the Smyth report describing in detail and plain language what fission was about”. In fact, the Americans were so keen on early publication of the Smyth report that on 12 August 1945 it was released for the Sunday morning newspapers³. So rather than a few months after Hiroshima, publication took place a few days before.

Jan H. J. Oelering

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1. *Nature* 376, 102 (1995).
2. Sherwin, M.J. *A World Destroyed* (Knopf, New York, 1975).
3. Groves, L. R. *Now It Can Be Told* (Deutsch, London).

Marine marinades

SIR — The letter from Milton Freeman (*Nature* 376, 11; 1995) on the dispute about whale species enriching Japanese rice bowls seems to report a major scientific finding, which should have deserved front-page coverage.

The author suggests that marination may have caused a DNA sequence to be identified as “midway between a minke and humpback”. To my knowledge, this is the first report of species hybridization by marination. New prospects for genetic engineering? Is that marinade recipe patented?

Berthold Heinze

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SIR — Freeman refers to an advertisement placed by this company in the 20 April issue of *Nature*, as well as our reporting of the whale DNA work of Baker and Palumbi. The advertisement in question was actually an older advertisement from the 15 September 1994 issue, which was used by *Nature* in order to meet press deadlines. The materials for the advertisement that we intended to place were unfortunately late arriving at the *Nature* offices.

Thus the copy in the printed advertisement did not contain information on more recent developments in Baker and Palumbi’s work. For example, last December, the US Fish & Wildlife Service ruled that wholly synthetic DNA made with the novel PCR procedure described in Baker and Palumbi’s paper is “not subject to per-

mitting or other requirements of CITES or federal conservation statutes, such as the Endangered Species Act” and further recognized “the contribution of DNA synthetics in wildlife forensic investigations”. The Fish & Wildlife Service deserves special credit for this regulatory flexibility, as its rapid endorsement of this novel PCR procedure enables US scientists to use a powerful molecular technique in studying and transporting the DNA of endangered species of flora and fauna.

John Hansen

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■ Further comments on this matter will appear in later issues of *Nature*.

Fewer notes

SIR — In a recent book review, the comment by Emperor Joseph II, “Too beautiful for our ears, my dear Mozart, and vastly too many notes”, was cited in connection with *La Clemenza di Tito* (1791) (ref. 1). Assuming it is authentic, this imperial observation actually refers to the earlier opera *Die Entführung aus dem Serail* (1782) (ref. 2). By the time of *Tito*, Mozart had achieved conciseness.

Martin F. Heyworth

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Fargo, North Dakota 58102, USA

1. Lock, S. *Nature* 375, 642–643 (1995).
2. Landon, H. C. R. (ed.) *The Mozart Compendium: A Guide to Mozart’s Life and Music* (Schirmer, New York, 1990).

Justifiable discrimination

SIR — I was interested to see (*Nature* 376, 202; 1995) that the Genetics Interest Group and others wish to give the government the powers to act against those found guilty of “genetic discrimination” in employment.

The discovery at the age of eight that I was colour blind, and hence that my chosen career of train driver was closed to me, was indeed a grievous blow. Other careers (such as airline pilot) were also closed, and although I never attempted it, my guess is that admission to art school might have proved dodgy. I have to say that I believe that all these discriminations are totally justified.

Alan D. B. Malcolm

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More precise solar-limb light-bending

A gargantuan exercise in data-analysis has produced the most accurate value yet of the deflection of radiation travelling near the limb of the Sun.

HISTORICALLY, the most dramatic proof that Einstein was on the right track with his general theory of relativity came in 1919, when Eddington and his associate Crommelin took themselves off to the western Pacific and the track of the total eclipse of the Sun in that year. They were bent on measuring the predicted deflection of light from distant stars lying near the limb of the Sun. As the world knows, the prediction was confirmed within the limits of the accuracy of the observations, to general applause. Einstein became a celebrity overnight.

What is not so well remembered is that the measurements in 1919 were not particularly accurate. The standard error of the measurements was roughly 30 per cent, while the displacement of the images of different stars in the field near the eclipsed Sun ranged from a half to twice the deflection Einstein had predicted. And later eclipse measurements have not been much better, largely because of the difficulties of making accurate measurements from the *ad hoc* observatories that have to be established.

Luckily, it is no longer necessary to wait for successive solar eclipses to improve on the Eddington result. More than 30 years ago, Irwin Shapiro (then and still at MIT) argued that the then-recently discovered radio-quasars would be more convenient and potentially more accurate ways of measuring the deflection of radiation near the limb of the Sun. If they happen to be in the sky, they have the advantage of being observable at all times, while those that happen to be in or near the Ecliptic will regularly come into near occultation of the Sun.

Shapiro's case is now well established. Since 1968, when the first radio results were published, the error of the measured deflection near the limb of the Sun has been steadily reduced, from something like 10 per cent to much less than 1 per cent. And, satisfactorily as it must seem, increasing accuracy has seen the estimated value of the deflection converge on Einstein's prediction. Is that not a sign that the further refinement of this test of general relativity can be safely put on the back burner?

Not so, and for good reasons, as will be apparent. The latest development is the use of a VLBI (for "Very Long Baseline Interferometry") technique for a direct measurement of the time delay in signals from radio-quasars passing near the limb of the Sun. Shapiro appears to have been

the first to point out in 1967 that the deflection of a light-beam would be accompanied by a delay, and that the two quantities are simply related to each other; it is pleasing that he is one of the authors of the new measurement, whose uncertainty appears to be 0.2 per cent (D. E. Lebach *et al. Phys. Rev. Lett.* **75**, 1439–1442; 21 August 1995). Those concerned are from the Harvard-Smithsonian Center for Astrophysics, the Haystack Observatory and from MIT.

The long baseline on which the measurement is based spans the distance between the Haystack Observatory and the Owens Valley Observatory in California. The target radio-source was the quasar 3C279; observations were made during an eight-day period on either side of the occultation of that source by the Sun in October 1987 (near the most recent sunspot minimum). To control for certain sources of systematic error, a nearby radio-source, 3C273B, separated by 10° on the sky, was used as a standard. Because of the need to observe at three different frequencies, two different radiotelescopes were used at each site. All four had to be switched between the target source and the control in synchrony, in practice on a 8-minute cycle.

The complexity of the data reduction evidently accounts for some of the delay in the appearance of this important result. (Another explanation is that Lebach's PhD thesis is cited as a source of further information.) Apart from the expected phase delay caused by the gravitational field of the Sun (not to mention that of the Earth), the effects of the Earth's atmosphere and interstellar plasma have to be allowed for. The three different frequency bands were, in reality, subdivided into fourteen, within each of which the signals received at the two sites were cross-correlated to yield estimates of the phase delay. It turned out to be necessary to allow for discrepancies between the atomic clocks at the two ends of the baseline, which is possible only by internal analysis of the data. Interestingly, one of the chief contributions to the eventual error of the measurement is the uncertainty about the relationship of the two sets of radiotelescopes to the Earth's axis, which inevitably affects the group's choice of a reference frame.

The conventional way of stating the outcome of both the time-delay and deflection measurements is in terms of a quantity which turns up in the predictions as $(1+\gamma)$,

where γ is a quantity calculated from the general theory of relativity to be exactly unity. Thus the deflection of a light-ray near the limb of the Sun turns out to be proportional to $(1+\gamma)/\delta$, where δ is the distance between the ray and the surface of the Sun as a fraction of the solar radius. The time-delay is similarly proportional to $(1+\gamma)$.

In each case, Einstein's relativity is verified if $\gamma = 1$ within the limits of error. Lebach *et al.* report that $\gamma = 0.9996 \pm 0.0017$, which is as much as anybody could ask for at this stage. But two of the authors, Shapiro and J. L. Davis, say that they now plan to work through the VLBI data collected in recent decades for geodetic purposes; they hope they can thus improve the precision of their data several-fold. (It is relevant that sensitive measurements of extra-galactic radio sources already make allowance for the Sun's gravity, even when the objects are a long way away from the Sun in the sky.)

What does any of this matter? There is more than academic interest in the search for a more precise γ . If, for example, should somebody seek to modify the general theory, he or she should ensure that the time-delay (or deflection) of light-rays near the limb of the Sun is properly accounted for. After all, the Brans-Dicke modification (invented in the 1960s by adding a scalar field to the ten-component tensor field of Einstein's space-time metric) was in part discarded on the strength of early radio-quasar measurements.

Yet these are still relatively local measurements. Moreover, the prediction of light-bending follows directly from Einstein's general theory in, so to speak, local mode, and does not depend on the choice of a particular solution of his equations to represent the Universe as a whole. If eventually modifications of general relativity are found necessary, they will surely be in applicable only where the gravitational field is much stronger than in the neighbourhood of the boring old Sun (which nobody has yet thought it worthwhile to designate as a black hole, although that may not take long the way things are going).

The crying need remains what it has been for the past two decades, that of marrying together general relativity and quantum mechanics. As things are, they are like chalk and cheese. It will be a great surprise if general relativity survives that marriage unchanged.

John Maddox

First steps on the ladder

Douglas J. Scalapino

ONE of the late Bernd Matthias's rules for finding new superconducting materials was "Never listen to theorists." On first sight this appears to be borne out by the report of the absence of superconductivity in the hole-doped 'ladder' compound $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$ by Hiroi and Takano¹ on page 41 of this issue. However, their finding that there is an insulator-to-metal transition on Sr doping of $\text{LaCuO}_{2.5}$, and the observation of a remnant spin gap in the doped metallic state, are in fact consistent with theoretical expectations.

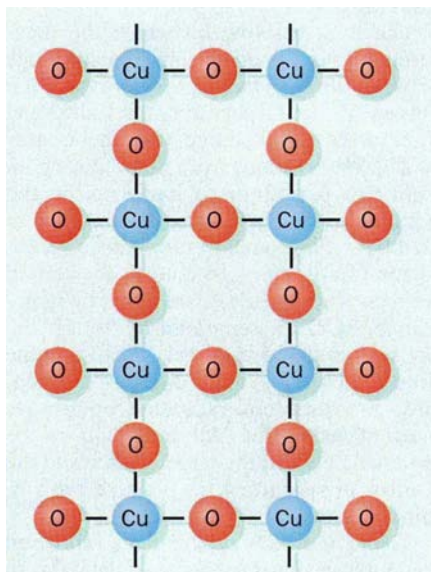
Ladder compounds, as the name suggests, contain chains of atoms connected by atomic rungs — here, both chains and rungs are of Cu–O. Various calculations^{2–10} for a two-chain spin ladder had suggested that it would be energetically favourable for the charge carriers to form singlet pairs leading to a spin-gapped metallic phase which could exhibit superconducting or charge-density-wave behaviour at low temperatures. Thus there is particular interest in the properties of $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$, the first known Cu–O two-leg ladder compound which has been successfully hole doped. In addition to the intrinsic interest in ladder compounds, there is also the more general hope that studies of such systems can provide new insight into the nature of the mechanism responsible for pairing in the high-temperature superconducting cuprates.

A schematic illustration of the structure of a two-leg Cu–O ladder is shown in the figure. Here Cu–O–Cu legs are connected by Cu–O–Cu rungs. In $\text{LaCuO}_{2.5}$, the ladders are characterized by Cu–O bond lengths which are less than 2 Å, whereas the bonds connecting different ladders are longer and oriented so that the coupling between ladders is weak¹. In the undoped $\text{LaCuO}_{2.5}$ material, the nominal valences of the ladder ions are Cu^{2+} and O^{2-} . The Cu^{2+} ion has one electron missing from a 3d shell, leaving the ion with a spin-½ magnetic moment. These spin-½ copper moments are coupled antiferromagnetically through an interaction J mediated by the oxygen. Thus the undoped insulator $\text{LaCuO}_{2.5}$ can be thought of as a two-leg ladder of antiferromagnetically coupled spin-½ magnetic moments.

In the actual material, the exchange coupling $J_{\perp}$ between the Cu spins across a rung is similar in strength to the coupling $J_{\parallel}$ between neighbouring Cu spins along the legs. But to provide a simple picture it is useful to consider the case in which $J_{\perp}$ is large compared to $J_{\parallel}$. In this case, Cu spins on either side of a rung will form a spin singlet in order to lower the energy of the system. It will then take a finite amount of energy, proportional to $J_{\perp}$, to

excite a rung to its triplet state. This excitation energy is called a spin gap, and leads to an exponential decrease in the magnetic spin susceptibility as the temperature is lowered until kT is below the spin gap energy. When holes are added by substituting some Sr^{2+} for La^{3+} , they go onto the ladders (that is, electrons are removed from the ladder) and within this simplified picture, pairs of holes tend to occupy sites on opposite sides of a rung so that the lowest number of the Cu^{2+} rung spin singlets are broken.

This provides an intuitive picture of the pairing; however, it is too extreme, and



Schematic illustration of the Cu–O ladders in $\text{LaCuO}_{2.5}$. When some of the La (not shown, but which occupies sites between the ladders, as shown in Fig. 1 of Hiroi and Takano's article) is replaced by Sr, hole carriers are doped onto the Cu–O ladders.

such a system, if it existed, would tend to phase-separate¹¹ into regions containing holes and regions containing spins. Numerical^{8,9} as well as analytical^{4–7,10} calculations on more realistic models in which the one-electron transfer t is larger than the exchange coupling $J = J_{\perp} = J_{\parallel}$ also find that the added holes tend to form pairs. These pairs have an amplitude for forming both across a rung and along the legs of the ladder. Furthermore, the relative phases of these amplitudes differ in sign^{4–6,8,9} (rather like the lobes of the d -wave pairing state proposed for the layered high-temperature cuprates). In the two-leg ladder, the gap for spin excitations remains for a range of doping but the gap for charge excitations vanishes, indicating that the doped system is metallic.

What is the nature of the dominant cor-

relations between the singlet hole pairs? One possibility is that the centre-of-mass momenta of the hole pairs are correlated; another is that the pair charge densities are correlated. The first of these possibilities corresponds to superconducting correlations, the second to charge-density-wave correlations. It turns out that both types of correlations are found in calculations for the doped two-chain ladder. In the actual material, the low-temperature phase that is realized is determined both by the relative strengths of these two types of correlations in a given ladder and by the relative strengths of the couplings between ladders. If the antiferromagnetic coupling along the chain $J_{\parallel}$ is equal in strength to that across a rung $J_{\perp}$, the charge-density-wave correlations along a chain decay more slowly than the superconducting correlations^{8,9}. For the superconducting correlations to dominate, one needs $J_{\perp}$ larger than $J_{\parallel}$ or a particularly strong superconducting coupling between the ladders.

Now Hiroi and Takano¹ find that the two-leg ladder compound $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$ exhibits an insulator-to-metal transition as x increases and holes are doped onto the ladders. Their measurements of the magnetic spin susceptibility show that the spin gap observed in the insulating $\text{LaCuO}_{2.5}$ material decreases but persists as the system is doped. This strongly suggests that some type of singlet pairing occurs. Furthermore, for $x = 0.20$, the conductivity is similar in magnitude to that of the layered high-temperature superconductor $\text{La}_{1.8}\text{Sr}_{0.2}\text{CuO}_4$ in its normal state.

These results are consistent with the expectations from model calculations made for a two-leg ladder with $J_{\perp} = J_{\parallel}$. As yet, however, there are no experimental measurements which provide information on the charge-density-wave correlations. These should occur with a spatial period set by the inverse of the doping fraction, x^{-1} . It may be that they can be more easily observed in a system in which some of the Cu has been replaced by Zn impurities which would act to pin the charge density wave. In addition, one might hope to observe effects of the 'd-like' symmetry of the hole pairs from the temperature dependence of the nuclear

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spin lattice relaxation times or from Raman scattering.

Knowing more about the pairing correlations in these new materials will tell us whether the existing theories of the doped spin ladders are adequate. Perhaps, if the theory is correct, ladders with shorter Cu–O–Cu rungs relative to the Cu–O–Cu sides can be produced. In this case one would expect that $J_{\perp}$ would be greater than $J_{\parallel}$, and this would be more favourable for superconducting correla-

tions. Beyond this, the contrast between the magnetic properties of insulating ladders with different numbers of legs in various materials, as well as the possibility of doping such materials, promises to open an exciting new area of materials science. $\square$

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LARGE-SCALE STRUCTURE

Evolution of galaxy clusters

J. Patrick Henry

THE report by Castander *et al.*¹ on page 39 of this issue of a new sample of clusters of galaxies is welcome news to astronomers studying the formation of structure in the Universe. The new sample, found as a result of X-ray emission, is generally fainter than previous results. It confirms and extends the somewhat tentative reports that clusters are evolving very rapidly. All these observations imply that the most massive gravitationally bound objects in the Universe are forming now.

There is good reason to think that the structure we see around us formed from small seed fluctuations in the otherwise extremely uniform mass density of the early Universe. The extra gravity of the seeds attracted the surrounding mass until the seeds grew to form the galaxies and clusters of galaxies we see today. Although this general idea is accepted, none of the details are known. In particular, it is not known when most of the structure of a given mass scale formed and what most of that mass is — ordinary baryonic matter or something more exotic.

Seeding

The seeds containing mass M are found by smoothing the density distribution over a distance $R = (3M/4\pi)^{1/3}$. The root-mean-square value of the initial seed density distribution of mass M , divided by the average density, is often taken empirically to be $\sigma(M) \propto M^{-(n+3)/6}$. The value of n is determined mainly by what constitutes most of the mass in the Universe. For a Universe with high average mass density, n is about -2.7 on cluster mass scales if neutrinos have masses of the order of 5 eV; it is around -2 if the Universe has a large vacuum energy density; and if all that exists is non-relativistic matter, then n will be about -1 .

Initially, $\sigma \ll 1$. An object forms when its seed achieves a density about twice the average (strictly 1.68 in most cases). That is, most objects of a given mass form at the epoch when σ for that mass first grows to near unity. The smallest masses form first,

so long as n is greater than -3 . We expect that most galaxies formed before most clusters of galaxies. Indeed, if structure does form in this bottom-up manner, the present will always be the epoch when the most massive objects are forming.

However, objects of any mass can form at any epoch, as for any value of $\sigma(M)$ there is always a finite probability that a given seed will have the necessary density. Constraints on the value of n , and hence on the composition of most of the matter in the Universe, can be obtained from whether structures of different mass form more or less at the same time ($n \approx -3$) or are widely separated in time ($n \approx 0$).

Astronomers can look back in time by looking out into space. If they examine objects at great distances the finite speed of light lets them see the Universe as it once was. Most galaxies formed so long ago that newly born examples are almost too far away to be observable with present instrumentation. Few, if any, galaxies have ever been observed being born and it will certainly be difficult to study the process.

Newly born clusters of galaxies should be closer and hence easier to study. Clusters have traditionally been found from identifying regional increases in the density of galaxies on photographic plates of the sky. This process has acknowledged deficiencies, not the least of which is the use of the human eye, a singularly non-objective instrument, to make most of the identifications. Consequently, most astronomers are chary of studies of cluster evolution using optically selected samples. X-ray selection offers a number of advantages which make such samples more trustworthy. Perhaps the most important point is that the X-ray gas is so hot that it would evaporate unless there were a deep gravitational well (in other words a cluster) confining it. An enhancement of galaxies, on the other hand, could result from a superposition along the line of sight of unrelated objects.

The results now obtained by Castander *et al.*¹ are the first from identifying the clusters in a faint sample of X-ray sources

discovered serendipitously in observations made with the German–US–UK satellite Rosat.

Previous studies^{2–4} of the evolution of clusters similarly selected by their X-ray emission showed that there were fewer high-luminosity objects in the very recent past (high luminosity should imply high mass). This work was at a barely significant level. Indeed, some of it² is now thought to have been a statistical fluke^{5,6}. So the first notable accomplishment of Castander *et al.* is to confirm that there were far fewer high-luminosity objects in the recent past, supporting the earlier idea that clusters seem to be forming now.

History

Nothing in astronomy is ever as simple to interpret as one would like. A cluster's X-ray luminosity depends on the temperature and density of its hot X-ray-emitting gas. Thus the evolutionary history of the X-ray luminosity depends on the history of the density seeds, convolved with the thermodynamic history of the hot gas. The second accomplishment of Castander *et al.* is to place some constraints on the hot gas history. The particular model ruled out is already known to disagree with local cluster data⁷, but other models have been proposed^{8,9}, and Castander *et al.* present one as well, which are all in agreement with these data. In fact, though, the combined data from the two faint X-ray surveys^{1,3,4} appear to be in conflict with this new model because a different n is required to fit the two samples.

Much work can and should be done to pin down the permitted combinations of gas and seed evolutions, but it may well be that the data will never unambiguously separate the two. A more promising approach, just now feasible using the Japanese–US satellite ASCA, is to determine the evolution of the cluster temperature. Any evolution will still be a convolution of that of the seed and temperature, but at least one variable, the gas density, will be eliminated. These studies, and other approaches, are beginning to convince cosmologists that they may attain their fond hope of determining how the magnificent structure we see about us came to be. $\square$

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Race to explain procrastination

Jeffrey D. Schall

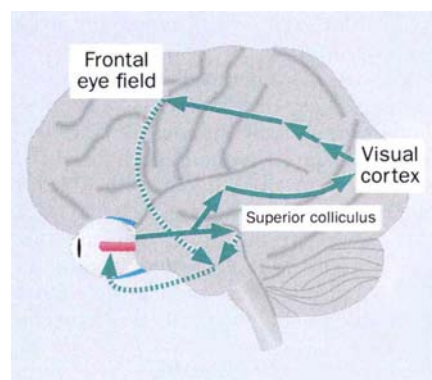
"WITH the speed of thought" is intended to convey the ultimate in speed. But when, in 1850, Helmholtz¹ clocked the velocity of signal conduction in the nervous system at a modest 50 metres per second, he recognized that mental processes could not be faster than brain processes. This discovery led to the birth of experimental psychology and the development of a new experimental paradigm — the idea was to measure the time taken by subjects to react to different stimuli under different instructions. How brain activity gives rise to behaviour could be understood by relating behavioural reaction time to the time taken by neural pathways to respond to the stimulus and generate the response^{2,3}. On page 59 of this issue⁴, Carpenter and Williams present evidence for a computational signal that may be a link between neural activity and behavioural reaction time.

When a light flashes in one's peripheral vision, the gaze shifts to look at it with a rapid eye movement called a saccade; the figure shows the brain structures responsible for generating visually guided saccadic eye movements. The neural response to a visual stimulus is conveyed from the retina to central structures such as the superior colliculus and primary visual cortex within 40–100 milliseconds. Visual signals are relayed to other cortical areas and subcortical structures that are ultimately responsible for generating the command to move the eyes. Brain structures such as the

superior colliculus and the frontal eye field that are responsible for generating saccades do so with a latency of about 30–50 ms. Thus, if the interval from the visual stimulus to an eye movement is due solely to conduction along the neural pathways between the retina and the extraocular muscles, then saccades should have latencies ranging from no less than 70 ms to no more than 150 ms. In fact, though, as Carpenter has pointed out, saccade latencies are typically longer and much more variable than this because the brain seems to procrastinate, probably because of the necessity to decide where and when to shift gaze⁵.

Just how the apparent procrastination arises from the nervous system is a mystery. The duration of processing in the brain, we now know, cannot be calculated by a simple addition of the time of axonal conduction and synaptic transmission between successive neurons. This is because no neuron single-handedly influences or is influenced by another neuron. Computations in the cerebral cortex are performed as each neuron integrates over time the weak and vacillating influences of hundreds of other neurons. The outcome of neural processing may be analogous to the coalition of votes among constituents racing to influence a decision.

Carpenter and Williams⁴ employ the concept of a race in the design of a simple stochastic model to account for both the



Simplified diagram of the brain circuitry responsible for generating gaze shifts. Solid arrows indicate the flow of visual signals into the brain. Dashed arrows indicate the flow of motor commands out of the brain. Saccadic gaze shifts can be generated by either the superior colliculus or the frontal eye field. The superior colliculus receives visual signals directly from the eye and sends a motor command to circuitry in the brainstem responsible for generating the saccade. In the cortical circuit, visual signals are relayed to visual cortex in the occipital lobe, whereupon multiple streams of processing lead to frontal cortex where the frontal eye field generates commands to move the eyes.

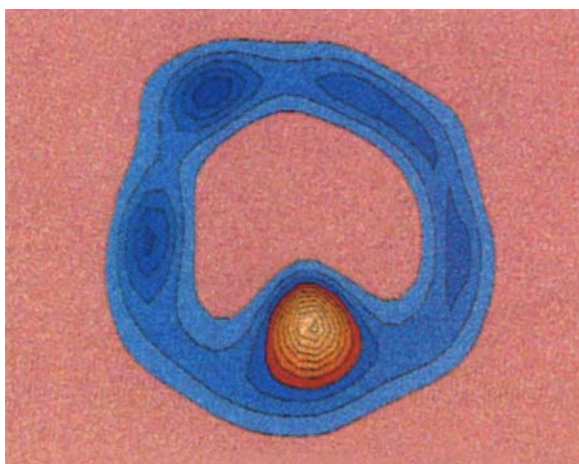
random and systematic variation in the latency to move the eyes when subjects are asked to shift their gaze to a spot of light that jumps to either the left or the right. Unlike typical neural networks with *ad hoc* elements and mysterious hidden units, this model is a mathematical formulation of a process underlying behavioural performance. And unlike many models of reaction time that seek to explain only central tendencies, this model is designed to account for the entire range and shape of the latency distribution. The model posits the existence of a signal that grows as information about the presence and location of a visual target accumulates. When this signal reaches a threshold, then a movement is triggered.

This general architecture is similar to other reaction time models⁶. In the model of Carpenter and Williams, the random variation of saccade latencies arises from stochastic variability of the rate of growth of the accumulator signal. These authors test the hypothesis that the accumulating signal may correspond to a likelihood ratio of the sort developed in theories about statistical decision processes. Theoretically, the likelihood ratio is an optimum measure on which to base decisions. To investigate whether likelihood ratio is a valid construct, they manipulated the decision process by systematically biasing the presentation of the targets, thereby demonstrating that when the probability of target presentation is changed, the latencies of subjects to shift their gaze to the visual targets varies as predicted by their model.

Clearly, further work should evaluate whether alternative models can simulate

Atom in a cage

Not abstract art, but concrete evidence of something that fullerene specialists had hoped and suspected to be the case, the figure shown here demonstrates that a metal atom can indeed be trapped within a cage of carbon. The contours map the electron density across a section through the metallofullerene Y@C₈₂, the shorthand notation for yttrium in an 82-carbon cage. The pink and



swollen bulge inside the circle's lower rim shows the high electron density expected to be associated with the yttrium atom — here clearly ensconced within the blue carbon framework. Theory and experiment had both suggested that this was so; but the new

structure, derived from X-ray powder diffraction experiments, may finally allay any doubts. Details of the experiment, carried out by Japanese researchers M. Takata *et al.* at Mie and Nagoya Universities, can be found on page 46 of this issue.

L. N.

these results. The model of Carpenter and Williams establishes a benchmark against which to judge competing hypotheses. What is more important, such models are necessary for us to understand how brain processes give rise to behaviour and mental processes. It is commonly said that the brain 'processes' information, but really, instead of directly monitoring information flow in the brain, recordings from neurons only measure changes in discharge rates. Neurophysiologists struggle with the question of what signal a neuron is carrying and therefore what its activity means.

Answers to this fundamental question can come about only through theories of the function of the neural circuit under investigation and its relation to behaviour. Such theories should make use of well formulated signals to perform the desired computations and also specify how those signals are conveyed in neural discharge patterns. For example, models of the oculomotor system use a concept known as 'motor error'. Models of sensory systems are based on the concept that neural activity encodes or represents attributes of sensory stimuli.

Several laboratories have started to investigate the activity of neurons thought

to be involved in perceptual and motor decisions⁷⁻⁹. An important implication of Carpenter and Williams' work is that likelihood ratio may serve as an effective measure of how much, over time, the activity of a neuron contributes to a decision. Linking hypotheses like this are needed to move beyond description towards explanation of mind-brain relations. □

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CLIMATE CHANGE

Warm days, hot nights

L. D. Danny Harvey

COMPUTER climate models driven by increasing carbon dioxide concentration predict that essentially the same warming should occur by day and by night. But this is not so. Between 1951 and 1990, records show that the average daily maximum of air temperature at the land surface has increased by only 0.28 °C, whereas the average increase in minimum daily temperature has been 0.84 °C — a factor of three larger. Why the discrepancy? Writing in *Atmospheric Research*¹, Jim Hansen and colleagues Makiko Sato and Reto Ruedy conclude that the mean warming and lower diurnal temperature range can only be explained simultaneously by a combination of regional increases in aerosol optical depth and low-level cloudiness, and a large-scale warming factor such as greenhouse gas increases.

This conclusion is not altogether unexpected. Regions and seasons showing the strongest reduction of diurnal temperature range also show statistically significant increases in cloudiness. Cooling due to industrially produced sulphate aerosols — either acting alone, or causing greater cloudiness — has been widely invoked to explain the discrepancy between model projections and observations, but so far only in qualitative arguments. Hansen *et al.*, in contrast, have used a three-dimen-

sional atmospheric general circulation model to examine how the diurnal temperature cycle is affected by increases in carbon dioxide, cloudiness and sulphate aerosols, acting individually and together.

Sulphate aerosols are a by-product of sulphur oxide emissions, which in turn are produced industrially from the combustion of sulphur-containing fossil fuels. The primary radiative effect of sulphate aerosols is to increase the reflection to space of solar radiation, an effect which will weaken daytime heating but have little effect on night-time temperatures. But sulphate aerosols also serve as nuclei for cloud condensation, and, by increasing the number of small droplets, could indirectly increase cloud reflectivity and the lifetime of individual clouds, thereby increasing mean cloudiness. Cloudier skies should be particularly effective in reducing the diurnal temperature variation, as they reduce both solar heating by day and infrared cooling by night. Observations indicating that cloud albedos are indeed higher where sulphate concentrations are large² and that increased cloudiness is associated with increasing sulphur emissions³ strengthen this connection.

Climate modellers distinguish between the 'equilibrium' response to a heating perturbation, which is the climate change

that occurs once the system has fully adjusted to the imposed perturbation, and the transient or time-dependent response. Hansen and colleagues find that, in equilibrium, a CO₂ increase capable of giving a mean warming of 0.5 °C reduces the diurnal temperature amplitude by only 0.08 °C averaged over all land. This is due in part to the direct increase in atmospheric infrared absorptivity caused by a higher CO₂ concentration and in part to the increase in atmospheric water vapour content produced by the CO₂-induced warming. The only realistic perturbation acting in isolation which can damp the diurnal cycle by the amount observed and change the mean temperature over land by 0.5 °C is an increase in middle-level cloudiness over land by about 3 per cent — but this produces mean cooling rather than warming. To match both the mean warming and the damping of the daily cycle, Hansen and colleagues find that the known greenhouse gas heating must be combined with increases in aerosol optical depth and low-level cloudiness which reflect the regionally varying increase in estimated anthropogenic sulphate loading in the atmosphere.

In transient response experiments, however, the picture changes. Aerosol and cloudiness changes, and the direct effect of CO₂ on atmospheric infrared absorptivity, quickly affect the diurnal cycle, whereas the change in mean temperature is delayed by two to three decades. This has two important consequences. First, CO₂ and aerosol increases acting together can explain a much larger fraction — between one-third and a half — of the observed damping in diurnal temperature variation than implied by equilibrium simulations. A corollary is that a smaller increase in mean cloudiness over land, around 1±0.5 per cent rather than 3 per cent, is required to explain the remaining damping. Second, and more important, as global mean temperatures come closer into balance with the greenhouse-gas heating perturbation (which must eventually happen), the difference between daytime and night-time warming can be expected to shrink. So, in the long run, daily maximum temperatures will increase by almost as much as the mean temperature. Nevertheless, to the extent that some effects of climate change depend on the rate of change of daily maximum temperature, a slower increase in daily temperature maxima could lessen the impact of global warming.

An important by-product of the effort to explain changes in the diurnal cycle is Hansen and colleagues' estimate that the combined cooling perturbation by aerosols and clouds is about half as large as the greenhouse-gas heating perturbation in the global mean. This result is independent of but consistent with attempts to calculate the relative aerosol and greenhouse-gas perturbations directly⁴. ▶

Confidence in this assessment of the relative importance of the different heating and cooling perturbations will be increased if the seasonal, vertical and geographical patterns of the response match observations well. A detailed fit between observations and model results may remain elusive, however. Day-to-night differences in warming over a broad region of the southwest tropical Pacific show a complex pattern, with some sites showing a decrease in diurnal temperature variation, some an increase and others no change⁵. A strong diurnal asymmetry is observed at lowland sites in the European Alps but not at high-altitude sites⁶, as one might expect if the cause stems from industrial sulphate. However, at a high-altitude site in the nearby French Pyrenees, daytime cooling and marked night-time warming is observed along with a significant (15 per cent) increase in cloudiness⁷.

Some of the changes in diurnal temperature structure could be a result of natural variability. In the climate model used by Hansen *et al.*¹, natural variability can produce regional changes in diurnal temperature range of around 1 °C, and changes averaged over all land of up to 0.2 °C, on a timescale of decades. To untangle the role of natural variability in the observed regional trends will take careful analysis of factors such as changes in cloudiness and soil moisture.

Another factor which could be influencing some observed changes is aerosol emission from vegetation burning at low latitudes, such as in central Africa and the Amazon Basin, which may have an effect on the regional radiation budget comparable to that of sulphate aerosols in middle latitudes⁸. Finally, recent decreases in the amount of stratospheric ozone appear to be strongly affecting temperature trends in the upper and middle troposphere⁹, thereby leading to changes in the vertical temperature structure which in turn could alter middle- and high-level cloudiness. According to sensitivity analyses¹, such cloud changes could also alter the diurnal temperature pattern.

In short, a meaningful comparison of climate projections with observed spatial and diurnal patterns of temperature change will require transient simulations using models with realistic geography, driven by concurrent changes in greenhouse gases and aerosols (both sulphate and bio-

genic), and including trends in stratospheric ozone. It will also require improvements in our ability to model cloudiness and in understanding the links between aerosols, cloud optical properties and cloud lifetime. Nonetheless, the work by Hansen and colleagues provides a broad quantitative explanation of current observations —

and further evidence that continued increases in greenhouse gas concentrations will lead to socially and ecologically important climate changes. □

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PHARMACOLOGY

Redirecting T-cell function

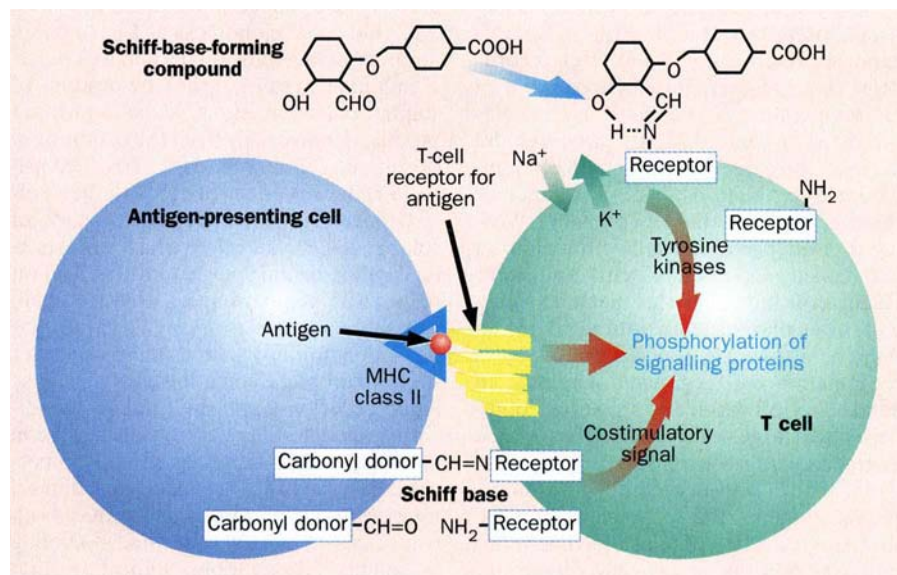
Gene M. Shearer

RECENT years have witnessed an increasing interest in the therapeutic potential of cytokines, the immunological hormones that coordinate immune responses¹. Cytokine-based therapy has been considered for a spectrum of diseases and conditions ranging from parasitic infections² and tuberculosis³ to cancer⁴ and AIDS⁵. But experience has shown that there are problems with systemically administering cytokines directly to patients, including toxicity, short half-life, and delivery to the immunologically active sites where they will be effective — the toxicity of interleukins 2 and 12, for example, has hindered their application in cancer^{6,7} and AIDS therapy⁸.

A way round these obstacles is described on page 71 of this issue⁹ by Rhodes *et al.*, who avoid direct cytokine intervention and instead introduce small Schiff-base-forming molecules that influence the production of endogenous cytokines. This strategy may circumvent

the need for the costimulatory signals normally required for the efficient activation of T helper cells and offers the potential advantages (yet to be demonstrated) of reduced toxicity and production of the necessary cytokines at the appropriate immunologically active sites (lymph nodes, for example) where the cells of the immune system congregate and communicate.

Although their study does not encompass all immunological parameters, the results of Rhodes and colleagues demonstrate the effects of Schiff-base-forming molecules in a diverse array of models. These include *in vitro* human and *in vivo* murine investigations of antigen-induced T-cell proliferation, virus-specific responses of cytotoxic T lymphocytes, cytomegalovirus inhibition, and arrest of the growth of solid tumours. Oral administration of one of these compounds to mice provoked an increase in production of interleukin-2 (IL-2) and interferon- γ , and



Promotion of Th1-type immunity by Schiff-base-forming drugs. A T-lymphocyte is activated when its receptor is occupied by a major histocompatibility complex (MHC) class II-bound peptide on an antigen-presenting cell. Costimulatory signals are triggered as a result of the formation of a Schiff base between a carbonyl group on the surface of the antigen-presenting cell and an amine group on the T cell. Rhodes *et al.*⁹ have tested a series of small Schiff-base-forming molecules (an example is shown here) that substitute for the natural carbonyl donor and provide a co-stimulatory signal to T cells by activating transport of K⁺ and Na⁺, leading to the phosphorylation of key signalling proteins.

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a decrease in the production of interleukins IL-4 and IL-6. This cytokine pattern is consistent with enhanced T-helper activity for generating a strong cellular immune response and a dominant type-1 or Th1-like cytokine profile⁵. Immunological parameters that still need to be investigated include IL-12 production and gene expression, natural killer cell activity and antibody synthesis — testing for these activities will give a more complete picture of the effects of these compounds on the immune system.

The Schiff-base-forming molecules appear to act by bypassing the costimulatory pathway that involves the CD28 protein on T-helper cells and B7-1 on antigen-presenting cells (see figure). The B7-1 costimulatory ligand is supposed to bias the immune system in the direction of strong Th1-like cellular responses, unlike B7-2, which may be more relevant for initiating Th2-like responses¹⁰. It will therefore be important to determine whether inhibition of the immune-potentiating effect of these compounds has any effect on the costimulatory pathway that can be blocked by anti-B7-2 antibody.

The demonstration by Rhodes *et al.*⁹ of the effects of Schiff-base-forming molecules in mice should allow this approach to be tested against direct cytokine infusion for toxic effects, functional half-life, stimulation of cytokine cascades and the homing of activity to immunologically important sites such as spleen and lymph nodes. The effects of these two approaches can also be compared against parasitic, bacterial and viral infections, as well as in the control of autoimmune diseases and induction of tumour rejection. Schiff-base-forming molecules might emerge as candidates for therapy in human diseases and conditions in which the loss of cellular immune function and reduced type-1 cytokine production are associated with disease progression. As noted in the paper⁹, some of these Schiff-base-forming compounds have been used for other medical purposes and have good bioavailability and low toxicity. Nevertheless, some work still remains to be completed before these molecules can be considered for clinical trials. □

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Zapping tandem SH2 domains

Arthur Weiss

TYROSINE phosphorylation is a frequently used means of communication between and within proteins in signal transduction pathways, achieved through binding of tyrosine-phosphorylated sequences to SH2 domains. SH2 domains are globular domains of about 100 amino acids which are expressed individually, in pairs, or in conjunction with other protein interaction domains (that is, SH3 and PH domains)¹. Each SH2 domain can function independently and binds tyrosine-phosphorylated proteins by using two adjacent binding sites — one site binds phosphotyrosine and a second determines the specificity of the interaction by recognizing adjacent residues. On page 32 of this issue², Hatada *et al.* solve the crystal structure of tandem SH2 domains of the protein tyrosine kinase ZAP-70 bound to a doubly phosphorylated peptide derived from the T-cell receptor (TCR) ζ -chain. The structure offers some surprises: not only does a coiled-coil loop appear unexpectedly between the SH2 domains, but a phosphotyrosine-binding pocket is formed from residues contributed by both domains, which lie close together.

ZAP-70 is a cytosolic protein tyrosine kinase expressed exclusively in T cells and natural killer cells³. It consists of two SH2 domains in tandem linked to a carboxy-terminal kinase domain. Another related protein tyrosine kinase, Syk, is more broadly expressed in the haematopoietic lineage. Both kinases participate in antigen-receptor signal transduction. The role of ZAP-70 in T-cell development and TCR signal transduction has been shown to be critical in studies of humans with a rare immunodeficiency syndrome resulting from mutations in ZAP-70 (ref. 4) and of mice deficient in ZAP-70 (ref. 5).

ZAP-70 interacts with the tyrosine-phosphorylated residues contained within the cytoplasmic domains of the non-antigen-binding subunits of the TCR, the CD3 and ζ -chains³. These tyrosines are found in sequence motifs termed ITAMs (for immunoreceptor tyrosine-based activation motifs), which are present singly in CD3 chains and as three copies in ζ -chains. ITAMs contain a YXXLX₍₆₋₈₎YXXL consensus sequence (where Y is tyrosine, L is leucine, and X is any amino acid) and are used by many types of antigen receptor. These motifs can confer a signal transduction function upon heterologous receptors.

The phosphorylation of both tyrosines in an ITAM, believed to be mediated by phosphotyrosine kinases of the Src family, such as Lck or Fyn in T cells, creates high-affinity binding sites for the tandem SH2 domains of ZAP-70. Once recruited to an ITAM at the plasma membrane, ZAP-70

can interact with and be activated by Src-family kinases as a result of transphosphorylation of the ZAP-70 kinase domain. The phosphorylation of ZAP-70 is a critical step for T-cell activation. So, getting ZAP-70 to the ITAMs at the plasma membrane is a key initial event.

The high-affinity binding of ZAP-70 to an ITAM depends on the phosphorylation of both tyrosines in the ITAM and on the binding function of both SH2 domains^{6,7}. This is consistent with a model in which both SH2 domains of a single ZAP-70 molecule are involved in binding to a single ITAM — it has been hard to detect any degree of interaction of either of the SH2 domains alone with a phosphorylated ITAM. These observations are in good agreement with mutagenesis experiments, which indicate that both tandem SH2 domains and both tyrosines in the ITAM are required for function.

Hatada *et al.* now reveal the molecular basis for this high-affinity interaction². The crystal structure shows that the two ZAP-70 SH2 domains interact when bound to a doubly phosphorylated ITAM and that the inter-SH2 domain forms a coiled-coil loop (see figure). The carboxy-terminal SH2 domain (SH2-C) binds to the amino-terminal (phospho)YXXL sequence of the ITAM according to previously established SH2-phosphopeptide ground rules, by means of a typical phosphotyrosine-binding pocket and a deep pocket that accommodates the leucine three residues further on. Likewise, the equivalent leucine in the more carboxy-terminal (phospho)YXXL sequence is bound in a conventional pocket by the amino-terminal SH2 domain (SH2-N). However, the amino-terminal SH2 domain has an incomplete phosphotyrosine-binding pocket. This phosphotyrosine-binding pocket is completed by residues provided by the adjacent carboxy-terminal SH2 domain. Thus, in order to bind both ITAM phosphotyrosines simultaneously, the two SH2 domains must interact to form a special type of phosphotyrosine-binding pocket.

How the SH2 domains interact is also quite interesting. There are only four hydrogen bonds at the interface of the two SH2 domains, suggesting a relatively weak intrinsic interaction between the SH2 domains. The rather impressive coiled-coil loop that is formed from the 65 residues between the SH2 domains may contribute to the overall stability of the SH2-SH2 interaction. In addition, the ITAM peptide interacts extensively with the surfaces of both SH2 domains and is likely to stabilize the interactions between the two SH2 domains.

It may be that the relationships between

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Deep heat

PRIMITIVE cave-dwellers, says Daedalus, enjoyed a useful domestic bonus: an even temperature. The thermal inertia of the surrounding rock gives deep caves a steady temperature, unchanging by day or night, winter or summer. So Daedalus wants to drag modern central heating forward into the stone age.

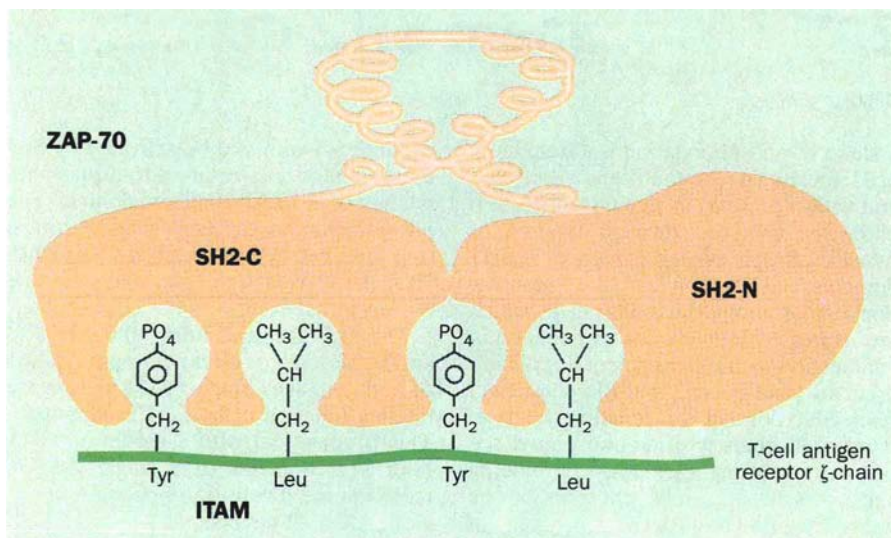
Imagine, he says, a heating system which pumped its water, not only through a boiler, but down through the rock deep underneath the house. In the depths of winter, ice-cold water pumped down through the rock would emerge usefully tepid, a good start to further heating. In summer, water heated by the torrid environment would return at the same equable temperature, which would then seem blessedly cool. But Daedalus goes further. The heated water pumped down during the summer would warm up the subterranean rock slightly. In winter, this added warmth would be returned. In a sense, the system would store a small sample of summer for release the next winter (and vice versa). Daedalus estimates that a region of rock perhaps 15 or 20 m in radius would make an adequate reservoir. The heat would be held mainly in its inner region, while the outer part acted as insulation.

Geological heat storage could be even more efficient with two reservoirs. Into one, you pump as much summer heat as you can, summer after summer; into the other you pump repeated samples of winter cold. Over the years, the reservoirs will stabilize at close to full summer and winter temperatures. Between them, they will meet all your heating and cooling needs, free.

The two reservoirs could be created by drilling two holes about 30 m into the ground in opposite oblique directions. The rock at the bottom of each hole would need to be fractured for maybe 10 m around, to provide a contact surface for circulating water. The technology should be trivial: the oil industry has already perfected ways of doing such things at vastly greater scales and depths. It might be more economic not to give each house its individual reservoirs, but to establish bigger ones that could serve an estate of houses, or even a whole community.

The tedious and expensive business of domestic heating would be transformed. The thermodynamic foolishness of burning fuel at 1,500 °C to provide heat at 25 °C would be ended, as would the complexity of domestic cooling. Vast amounts of fuel would be saved and carbon dioxide emissions cut back. Sadly, global warming might gradually sabotage the scheme by slowly reducing the cold of winter.

David Jones



Recognition of antigen-presenting cells by T lymphocytes is mediated by the T-cell antigen receptor (TCR). Stimulation of the TCR causes its immunoreceptor tyrosine-based activation motifs (ITAMs) to become phosphorylated so that they can bind a signalling protein, ZAP-70, inside the cell. ZAP-70 is a protein tyrosine kinase that associates with the ITAM on the TCR ζ -subunit through its SH2 domains in a highly selective arrangement revealed by the crystal structure solved by Hatada *et al.*²

the two SH2 domains and the inter-SH2 coiled coil do not exist in the unbound state, so a major allosteric change in ZAP-70 might occur as the SH2 domains bind the doubly phosphorylated ITAM. Several consequences are possible, but one of the most interesting is that this change could influence other domains of ZAP-70. Indeed, Hatada *et al.* suggest that the coiled-coil region may interact with the kinase domain to alter its function.

Although there is no evidence yet that ZAP-70 kinase activity is altered by ITAM binding, the activity of the related kinase Syk increases when bound to an ITAM⁸. A similar regulation of catalytic activity resulting from the engagement of SH2 by tyrosine-phosphorylated sequences has been proposed for other proteins containing tandem SH2 domains, such as the p85 subunit of phosphatidylinositol-3-OH kinase⁹ and the tyrosine phosphatase SH-PTP2 (ref. 10). In the case of p85, the inter-SH2 domain appears to be important in activating the catalytic function of the p110 subunit of phosphatidylinositol-3-OH kinase¹¹.

From this structure we have learned more about the ways in which SH2 domains can interact with phosphotyrosine, and as a result will need to think differently about domains that are linked in tandem in other proteins. There is much more concerning ZAP-70 activation and function to be explored, but the potential for allosteric change has interesting implications. For instance, ZAP-70 is not always phosphorylated or activated when bound to the phosphorylated ζ -chain. ZAP-70 is constitutively bound to a phosphorylated ζ -chain in thymocytes and lymph-node T cells and is not phosphorylated or activated until the TCR is stimulated¹². In anti-

gen-specific T cell clones and hybridomas, ZAP-70 binding to the ζ -chain is induced by altered peptide ligands but ZAP-70 is not phosphorylated, in contrast to the phosphorylation induced with agonist peptide antigens^{13,14}.

What both of these situations have in common is a different pattern of ζ -chain phosphorylation. The ζ -chain contains six tyrosines in the three ITAMs, so different patterns of phosphorylation of these tyrosines could present different options for binding ZAP-70 SH2 domains to a phosphorylated ζ -chain. These would result in distinct allosteric changes in ZAP-70, perhaps altering the structure of the inter-SH2 domain. Finally, the structure solved by Hatada *et al.* may eventually form a basis for the design of immunosuppressive drugs that target weak links in the ZAP-70 structure. □

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A shopping trolley seismograph

SIR — On 17 January 1995, a devastating earthquake struck the port city of Kobe in western Japan, leaving 5,502 people dead and 2 missing. The catastrophic scenes were recorded at many 'convenience' stores by closed-circuit video cameras. These video records (kindly provided by K. Nishimura of the Japanese Broadcasting Corporation (NHK) Kobe office) enable us to estimate ground motion and thereby to infer the nature of the buried fault beneath Kobe. Its precise location is still vague, although the southwestern part of the fault was traced along a pre-existing fault on Awaji Island (Nakata, T. *et al. J. Geogr.* **104**, 127–142; 1995). The convenience stores usually use basket-stands with castors which can move

in any direction on the floor. According to the inertia law, the basket-stands must move in the opposite direction to the motion of the floor, allowing their use as a form of seismograph.

A typical scene appearing on the video display is as follows. People first noticed the P-wave arrival through the vibration of the floor and other objects. A few seconds later, a large horizontal motion occurred. This later motion is interpreted as the arrival of the S wave. Soon after this, a larger shock hit stores. This larger shock can be interpreted as the direct effect of the fault slip beneath the stores.

The basic idea for inferring the surface trace of the buried fault is illustrated in Fig. 1. There are three theoretical patterns of ground motion depending on the location of the stores relative to a propagating, right-lateral fault: (1) on the north side of the fault the ground moves towards the northwest and then northeast, effectively moving clockwise; (2) on the south side of the fault the ground moves towards the northwest and then southwest, effectively moving anticlockwise; (3) at the extension from a fault tip the ground moves toward the northwest only.

The actual ground motions were more complex than these simple patterns, probably due to heterogeneous slip on several fault segments. Moreover, video records were often stopped immediately after the S wave's arrival because of the interruption of the electricity supply. In spite of these difficulties, these basic patterns were identified for nearly half of the video records obtained. In addition to these data, two more sets of data are available. One is the video record at the Kobe office of NHK. The other is GPS

data at Kobe University. Both locations are inferred to be on the north side of a fault trace. The strong-motion instrument at Kobe JMA (Japan Meteorological Agency), which is a little west of NHK, also recorded the ground displacement. It roughly indicates a clockwise motion following the arrival of the lateral S wave.

The ground motion patterns are summarized in Fig. 2. The video data alone cannot well constrain the northeastern part of the fault trace. For this part the information obtained from teleseismic body-wave analysis was used, where three sub-events with different fault orientations were derived (Kikuchi and Kanamori, unpublished data). The fault strike of the third sub-event, which is located in the Kobe area, is $N60^\circ E$.

This study shows that the earthquake fault co-seismically reached the area beneath Kobe and that large opposite ground motions occurred at two adjacent points, only 1 km apart (NHK and the southern point). This enables us to pinpoint the earthquake fault beneath Kobe (Fig. 2). The fault trace (that is, the intersection of the fault plane with the surface) runs between the heavily damaged zone and the aftershock zone.

Thus, video records at convenience stores can be very usefully employed as a form of seismogram. They also contain useful information about human behaviour, providing us with lessons on how to protect ourselves during sudden, strong earthquake shocks.

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Olfactory receptors guide axons

SIR—Subsets of olfactory receptor neurons (ORNs), each expressing a particular type of receptor protein^{1,2}, are broadly distributed in the sensory epithelium^{3,4}; yet members of each subset send their axons to only one or a few of some 2,000 modules, called glomeruli, in the olfactory bulb^{5,6}. The basis for this specific axon targeting is unknown.

A clue has come from correlated mutation analysis, which has identified residues in the receptors that may be critical for binding odour molecules⁷. This has supported previous evidence that odour molecule determinants interact differentially with residues within a transmembrane binding pocket in the receptors⁸. In addition, we have found that specific residues in the second extracellular loop (E2) show correlations with critical residues in the binding pocket (see figure), implying a functional relation between these residues in the loop and the binding pocket^{7,9}.

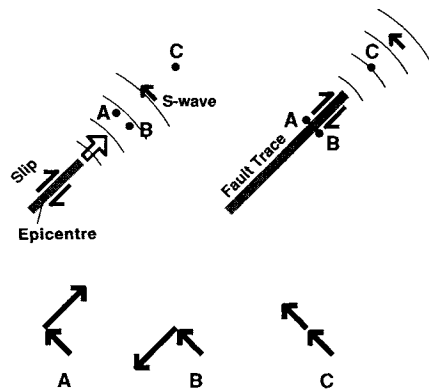


FIG. 1 Idealized ground motion associated with right-lateral fault propagation. Points A and B are located on opposite sides of an extending fault trace, while C is beyond the fault tip. These points were all shaken to the northwest by S waves generated from the epicentre, and then subjected to different motion depending on their location relative to the fault trace. The combination of S wave and the subsequent motion gives three different patterns of ground motions as shown at the bottom.

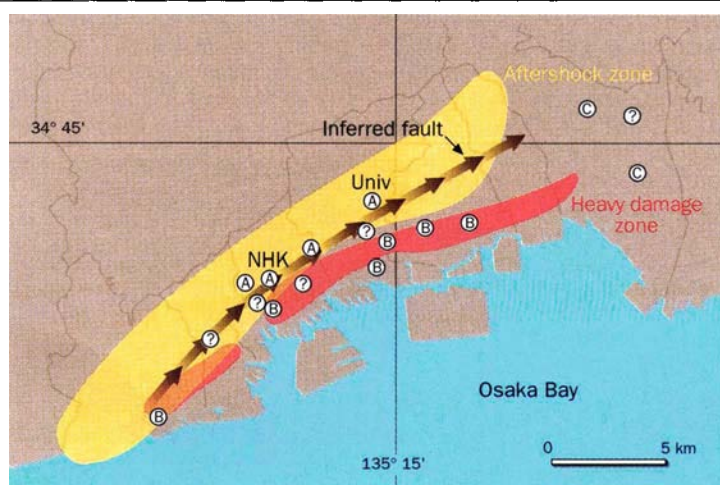
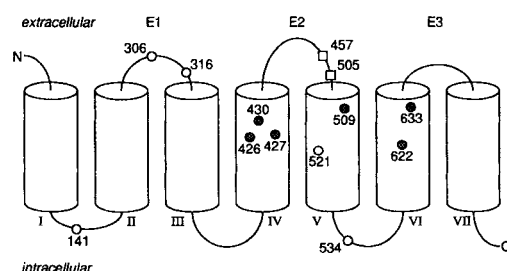


FIG. 2 Map of the Kobe area. Ground motion patterns (A, B and C) identified by the video data are plotted at the sites of the convenience stores. 'Univ' denotes Kobe University; '?' indicates that the pattern cannot be identified because the video records were stopped immediately after S-wave arrival. The solid line with arrows indicates a fault trace inferred from these data as well as the teleseismic waveform analysis.



Correlated mutations in rat olfactory receptors (see also ref. 7). Transmembrane α -helices (denoted by roman numerals) are shown as cylinders, interhelical loops as lines. E1, E2 and E3 indicate respective extracellular loops. Squares indicate correlated E2 residues; shaded circles indicate predicted ligand-binding residues; open circles indicate correlated residues of unknown function.

Correlated residues	r_{ij}	
316	457	0.973
430	457	0.973
505	509	0.997

Significant pairwise correlations with E2 residues. r_{ij} is the correlation coefficient for pairwise comparison of sequence positions i and j . Correlations were calculated by the CORMUT option of WHATIF¹⁵.

The potential importance of loop E2 has already been suggested by the finding of nonrandom amino-acid substitutions resulting from positive selection¹⁰, implying that loop E2 has a function unique to each receptor subtype. Also, a survey of the olfactory receptor family by hydrophobicity profiles¹¹ has shown that loop E2 contains approximately 35 residues, substantially more than most other members of the G-protein-coupled receptor superfamily.

Among possible functions for loop E2, interactions with odour molecules seem less likely because the hydrophilic loop and aqueous environment provide a poor binding site for odour molecules, which for terrestrial mammals are typically hydrophobic². Interactions with odour binding proteins (OBPs) or chaperonins are also less likely because they appear inconsistent with the extreme sequence diversity in the loop and the apparent functional specificity of each subtype (see ref. 10).

A more likely possibility is raised by the

recent finding that messenger RNA for putative olfactory receptors is present in the axon terminals of ORNs within their target glomerulus^{5,6}, suggesting that the receptor molecules may be involved in guiding axons to their target glomeruli. We propose that loop E2 contributes to this through axon-to-axon or axon-to-substrate interactions. Sequence motifs in the loop, especially conserved cysteines, may mediate this function; similar motifs are present in integrins and growth factors.

The correlated mutation analysis⁷ shows a one-to-one correspondence between the residues at positions 457 (loop E2) and 430 (predicted odour binding site; see table in figure legend). Thus, the residue at position 457 carries sufficient information to infer the residue at position 430 (Lys:His, Gln:Gln, Asn:Lys and Val:Ile, respectively). Positions 505 and 509 form another such pair. We anticipate that additional correlated pairs will be revealed by analysis of further receptors. Loop E2 can therefore function as a cell-surface identifier for the receptor subtype, reflecting the specificity of the odour-binding pocket. Consistent with this is our observation that no two olfactory receptor subtypes share the same loop E2 sequence^{12,13}.

The variation of loop E2 residues across

subtypes may contribute to the abilities of ORN axons of one subset to find their common target glomerulus, and of related subsets to target neighbouring glomeruli. This could provide a basis for the functional topography of the olfactory bulb. The loops could form homophilic interactions between axons, or bind preferentially to specific receptors, adhesion, or chemotactic molecules. Other extracellular domains (E1 and E3) could participate in these interactions, but they do not seem to share the special properties of loop E2, namely the hypervariability, positive selection, uncommon length and cysteine motifs.

Immunocytochemistry has suggested the presence of the olfactory receptor protein in the ORN axons (R. Reed, personal communication). Furthermore, receptor mRNA is evident in ORNs before their axons reach the olfactory bulb and establish synapses¹⁴, consistent with a role for the receptor in development.

Olfactory receptors may perform a dual role, as odour receptors in the sensory cilia and as guidance molecules in the axons. This may represent a more highly specific mechanism of axon guidance than any previously identified and implies a novel property for some members of the G-protein-coupled receptor superfamily.

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Cell damage by near-IR microbeams

SIR — Continuous-wave laser microbeams are generally used as diagnostic tools in laser scanning microscopes or, in the case of near-infrared microbeams, as optical traps for cell manipulation and force characterization^{1,2}. Because single-beam traps are created with objectives of high numerical aperture (NA), typical trapping intensities and photon flux densities are, respectively, of the order of MW cm⁻² and 10²⁷ cm⁻² s⁻¹. These extremely high fields may induce two-photon absorption processes³⁻⁵ and anomalous biological effects.

We have investigated these phenomena by studying optically trapped motile human spermatozoa maintained in HEPES-buffered fresh human tubal fluid labelled with a live/dead fluorescence assay kit (Molecular Probes). Trapping effects at 760 and 800 nm (70 mW) were compared using a tunable, continuous-wave Ti:sapphire laser coupled to an inverted fluorescence microscope. Visible two-photon-excited intracellular fluorescence was detected with a cooled CCD camera.

Viable cells showed green fluores-

cence (515 nm) corresponding to the membrane-permeable live-cell probe SYBR 14 (Fig. 1). Damaged cells showed red fluorescence (636 nm), due to intranuclear accumulation of the membrane-impermeable dead-cell probe propidium iodide. Figure 1a shows a brightfield image illustrating sperm structure, flagellar motion and an intense green spot in the centre of the sperm head. Without brightfield illumination, the sub-micrometre spot persisted

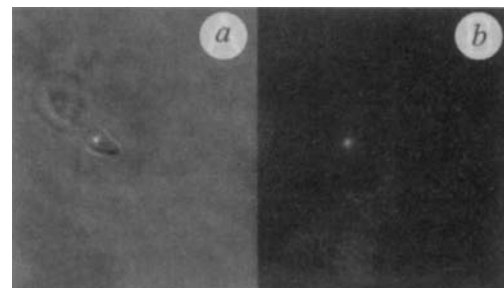


FIG. 1a, Brightfield image of a motile human sperm cell confined in the 760-nm optical trap. Two-photon-excited fluorescence of a live/dead fluorescent probe is clearly visible as an intense sub-micrometre spot in the 5- μ m-long sperm head despite the presence of halogen lamp illumination. **b**, The two-photon-excited fluorescent spot is more clearly visible in the absence of brightfield illumination.

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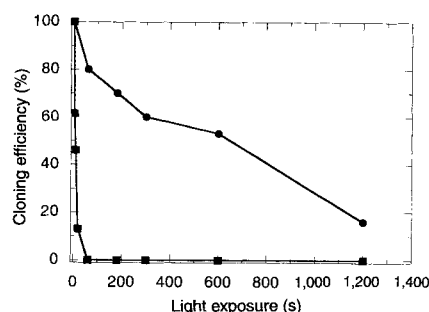


FIG. 2 Cloning efficiency against exposure time for CHO cells; 88 mW of 760-nm (■) and 800-nm (●) microirradiation.

and became red within 65 ± 21 s ($n=10$). This shows two effects of the 760-nm trapping beam. First, two-photon-excited visible fluorescence is induced by the near-infrared trapping beam. Second, green to red fluorescence changes (coinciding with cessation of flagellar motion at 35 ± 20 s ($n=10$)) indicate that the trap can cause lethal damage. In contrast, we were able to maintain most sperm ($96 \pm 6\%$, $n=50$) in an 800-nm trap for 10 min without either loss in motility or intranuclear propidium iodide accumulation.

Unlabelled spermatozoa showed blue, two-photon-excited autofluorescence which became 10–100-fold brighter within 600 s of 760-nm trapping ($n=10$). Autofluorescence was also detected during 800-nm trapping, but the spot intensity did not change. As autofluorescence signals are probably due to reduced pyridine coenzymes (NAD(P)H), 760-nm alterations may be a consequence of UVA-like, two-photon-induced perturbations of the cellular redox state (that is, oxidative stress).

A final indicator of cellular damage was provided by evaluating the cloning efficiency of microirradiated Chinese hamster ovary (CHO) cells. Interphase cells exposed for up to 1,200 s (88 mW) and maintained in an incubator for 5–6 days were considered to be unaffected by light if clones consisting of ≥ 50 cells were produced. Cells irradiated with 760 nm were unable to form clones following light exposures of ≥ 60 s, whereas cloning efficiencies of 80% and 50% were measured following 60- and 600-s exposures at 800 nm (Fig. 2).

In conclusion, continuous-wave near-infrared microbeams can stimulate multiphoton processes in single living cells. The consequences of microirradiation include

membrane permeability changes, alterations in cloning efficiency and UVA-like stress. These processes are clearly wavelength-dependent and should be considered during trapping experiments, particularly when using short-wavelength, near-infrared lasers.

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Kaposi's sarcoma in pregnant women

SIR — Lunardi-Iskandar *et al.*¹ demonstrated that the β -chain of human chorionic gonadotropin (hCG) kills Kaposi's sarcoma-derived cell lines in culture and inhibits tumour production by these cell lines in nude mice. They also observed regression of Kaposi's sarcoma in two women during pregnancy, when levels of this hormone are elevated². Because of the potential aetiological and therapeutic significance of these results, we examined clinical data from the Dermatovenereology Clinic, University Teaching Hospital, Lusaka, Zambia. We postulated that if hCG protected against disease in humans, AIDS-related Kaposi's sarcoma would occur with lower frequency or severity during pregnancy.

We determined the pregnancy status of all female patients ≤ 40 years old who were newly diagnosed with AIDS-related Kaposi's sarcoma during 1994. For comparison, we used new cases of venereal ulcer or discharge presenting during 4 months (January, April, July and October) of that year. Non-pregnant women with Kaposi's sarcoma were further classified into those with and without a child under 2 years of age.

Thirteen (15%) of the 84 young women with Kaposi's sarcoma and 25 (19%) of the 133 young women with venereal disease were pregnant at the time of presentation. Adjusted for age, the odds ratio for the association of current pregnancy and Kaposi's sarcoma was 1.0 (95% confidence interval, 0.4–2.1). An additional 23 (27%) women with Kaposi's sarcoma had a child under 2 years of age. Thus, 36 (43%) of the Kaposi's sarcoma patients had been exposed to elevated levels of hCG within the past 2 years.

We also assessed the relationship of pregnancy status to extent of tumour at presentation, classified as localized against disseminated according to ref. 3. Seven (54%) of the 13 who were pregnant had disseminated disease, as compared with 12 (52%) of those with children under 2 years of age and 19 (40%) of the 48 who were neither pregnant nor had a

child under 2. The age-adjusted odds ratio for the association of current pregnancy and disseminated sarcoma was 1.3 (95% confidence interval, 0.4–4.2).

These clinical data do not support the prediction from laboratory results that hCG is protective against Kaposi's sarcoma. Our data are not adjusted for possible fertility differences between these patient groups, nor for possible referral bias due to pregnancy and/or antenatal care. Furthermore, lack of effect at physiological levels does not exclude efficacy at higher pharmacological levels, for which the best test is a randomized, controlled clinical trial. Nevertheless, as pregnant and non-pregnant women appear to have a similar risk of this disorder, it is likely that factors other than hCG explain the difference between men and women in incidence of Kaposi's sarcoma.

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SIR — The classical pregnancy hormone and tumour marker human chorionic gonadotropin (hCG) has long been known for its ability to sustain pregnancy. In addition, it acts as a survival factor by suppressing apoptotic cell death in reproductive organs⁴. An entirely adverse effect of hCG, its blocking ability on tumorigenesis and metastasis of Kaposi's sarcoma, has recently been reported¹. The authors' hypothesis that the trophic hormone hCG and its free β -subunit (β hCG) cause regression of Kaposi's sarcoma was tested in immunodeficient Bg-nude mice as well as *in vitro* with pregnancy sera of human and mouse origin, and with different preparations of hCG-derived molecules. Their premises run contrary to several long-held concepts and definitions of endocrinology concerning the evolutionary origin, biological function and nomenclature of hCG-derived molecules.

From an evolutionary point of view, hCG is a young hormone, as the genetic events generating its β -subunit took place long after the lineages of rodents and primates diverged⁵. Rodents possess neither a β CG gene nor a placental hormone similar to CG (refs 6, 7). In consequence, the mouse displays a gestational profile of hormone dependency entirely distinct from humans. Human gestation is dependent on placental hCG right from the beginning, whereas for the maintenance of pregnancy in rodents pituitary-derived luteinizing hormone (LH) — the cognate molecule of hCG — is an indispensable stimulus only in the second trimester³. The question remains, what is causing the

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effects on Kaposi's sarcoma in the absence of a CG-like molecule in mice?

Of potentially more importance, the biological function of hCG is mediated by the α hCG/ β hCG dimer and not by the free β hCG subunit, but the same function and receptors are being proposed for hCG β as for hCG by Lunardi-Iskandar *et al.* This contradiction could be clarified by conventional radioreceptor assays or by direct demonstration of LH/hCG-receptor gene expression, but not by an immunohistochemical demonstration of receptors with a polyclonal antiserum against the ligand, as performed by Lunardi-Iskandar *et al.* The hormonal profiles of pregnancy sera differ vastly from sera of non-pregnant humans and mice, not only with respect to hCG. The investigators should have included some conclusive specificity controls such as immunoneutralization by monoclonal antibodies against both hCG and its subunits^{9,10}. The same applies to the crude hCG preparations used in their bioassays, especially as they did not rely on highly purified material (for example, hCG CR-127, 14,900 IU per mg), which is made available by their own research institution, NIH. Whether it is really CG, or even more surprisingly free β hCG, which exerts antioncogenic effects on Kaposi's sarcoma is therefore not resolved.

Finally, by definition, β hCG is not a pregnancy hormone, as claimed by Lunardi-Iskandar *et al.* It is at best a pregnancy marker whose serum levels are at least 100–1,000-fold less during gestation¹¹. Furthermore, a "purified native hCG preparation containing 80% β hCG and 20% α hCG" is a contradiction in terms and certainly not a biologically active holo-hormone.

We believe that hCG-like molecules

have a potential for additional functions, especially as β hCG and alternative messenger RNA splice products with open reading frames have been shown to be expressed, not only in the placenta, but also eutopically in the testis¹². Moreover, the main metabolic product of hCG, the β hCG core fragment, has a striking structural similarity to nerve growth factor and other members of the superfamily of 'cystine-knot' growth factors, suggesting a distinct biological function¹³. The suggestions of Lunardi-Iskandar *et al.* are, however, not yet proven.

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LUNARDI-ISKANDAR *ET AL.* REPLY — Rabkin *et al.* state in their analyses the interesting observation that pregnancy does not protect against Kaposi's sarcoma (KS). We agree that it is likely that factors other than hCG explain the male/female differential in this disease. In pregnancy, hCG levels peak only for a short period, and even that peak would be insufficient for long-lasting effects. Rather, we propose LH, and not hCG, as a candidate for a hormonal contribution to the sex differential in Kaposi's sarcoma¹, because the LH β -chain is 85% homologous to the β -chain of hCG^{14,15}, and because LH is higher in women than in men and much higher at some stages of the menstrual cycle¹⁶. Moreover, we find that LH has some of the same effects we reported for some hCG preparations (unpublished results).

Berger and Dirnhofer are concerned that because mice do not have hCG, but do have LH-like molecules, the inhibitory factor in mouse sera cannot be the same as in humans, namely β hCG. Mice may not have the corresponding β CG gene nor a placental hormone similar to CG, but sera of pregnant mice and women have an anti-KS factor, especially in early pregnancy. This activity is present in some preparations of hCG; it resides in the β -chain, as demonstrated using purified native β hCG¹ and also purified β hCG recombinant peptides (our unpublished data). Berger and Dirnhofer note that LH carries a related activity. Because of the homology between β hCG and β LH, we assume that if mice do not have CG, the anti-KS activity in mouse sera may well be LH or LH-like.

They also state that the reproductive biological effects of hCG are associated with the dimer. This is indeed the case; however, several workers have shown that the β -chain alone can induce signal transduction^{6,17–19} and also that β hCG competes with the dimer for receptor binding¹³, so there is good evidence that the β -chain alone contains some biological activity.

With respect to the presence of hCG

receptors, all our preliminary results indicate that the Kaposi sarcoma cells have hCG receptors (unfortunately, there was a mistake in our figure legend: the polyclonal sera were not to the ligand but to the receptor), but it is possible that the mechanism for the effect of β hCG could be independent of these. Berger and Dirnhofer note the similarity of β hCG to some growth factors²⁰, and we believe that one interpretation of our results (N. W. Isaacs, personal communication) is competition of β hCG for a growth factor needed by these tumour cells.

We disagree with Berger and Dirnhofer's contention that results with monoclonal antibodies to hCG would be more conclusive than those presented. We feel that the use of purified native β hCG and purified recombinant peptides of β hCG is sufficiently strong evidence to draw the conclusions made in our paper.

Finally, let us reiterate the novelty of our paper: Kaposi's sarcoma Y-1 cells are the first proven malignant cells obtained from HIV-associated Kaposi's sarcoma and they are killed by purified β hCG peptides. A possible additional novelty is the hypothesis of a gender susceptibility difference based on LH.

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Erratum

IN the Scientific Correspondence "Non-invasive bird tagging" by D. Michard, A. Ancel, J-P Gendner, J. Lage, Y. Le Maho, T. Zorn, L. Gangloff, A. Schierer, K. Struyf and G. Wey (*Nature* 376, 649–650; 1995), the reference list was inadvertently omitted. It is given below. Also, the surname of Alfred Schierer was misspelt.

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Usage and abuse

Ryan J. Huxtable

Consuming Habits: Drugs in History and Anthropology. Edited by Jordan Goodman, Paul E. Lovejoy and Andrew Sherratt. Routledge: 1995. Pp. 244. £35, \$49.95.

Drugs and Narcotics in History. Edited by Roy Porter and Mikulás Teich. Cambridge University Press: 1995. Pp. 227. £30, \$49.95.

COCA-COLA has "something other than just the taste; the accumulated memory of all those ball games and good experiences as children which coke was a part of". This quotation from the *Wall Street Journal* epitomizes the complexity of issues concerning the use of psychoactive substances. The original Coca-Cola, of course, contained cocaine from the cola (*Erythroxylon*) extracts used in its formulation. This psychoactive alkaloid has long vanished from the drink, to be replaced by another psychoactive substance, caffeine, but the tenacity of Coca-Cola devotees was shown by the uproar when the formula was changed in 1985. New Coke had to be withdrawn, even though most drinkers, tested objectively, could not distinguish between the old and new cokes. But to change the formulation was to rob consumers of a comforting echo of their childhood.

This example points to the impossibility of considering the use of psychoactive substances from a solely pharmacological viewpoint. Their use incorporates social, economic, legal and public health aspects, and is associated with rituals and ceremonies that help to organize and structure the users' lives. Ritual can extend from the use of wine in the Christian mass or tea in the Japanese tea ceremony at one end of the spectrum, where the use of the psychoactive agent becomes secondary to the occasion, to the recent US creation of a coffee ceremony at the other, where the thing-in-itself is the central point. This movement, originating in Seattle, has developed its own language (who in England — or Italy — would know the difference between 'wet' and 'dry' cappuccino?) and involves paying vast premiums for complex preparations such as a double mocha latte with whipped cream and nutmeg. Following a trip to Italy, I told my local coffee vendor that I had been in the land of true cappuccino. "Really?" he asked. "You just got back from Seattle?" This increasingly ritualistic US pastime is still centred on the psychoactive nature of coffee, as shown by the advertisement of one company: "After my morning cup of Bad Ass coffee, I go to the park and feed the birds... to my cat".

Consuming Habits is intended to encourage research on the anthropology of psychoactive substances and on the historical and cultural contexts of their uses. Although punning book titles are usually off-putting outside fictional works such as

Finnegan's Wake, the two senses of habits of consumption and of habits that consume the user are equally appropriate in the title of the book under consideration. Although it derives from a conference, containing ten chapters by various authors, the book avoids the usual drawbacks of the genre. The complexity of the issues is suggested not by any integrated attempt to cover the whole field but by case studies of selected substances in selected societies. The first five chapters are concerned with traditional societies outside Western Europe, and the last five with the economically interdependent societies that have arisen since the increase in global trade relationships of the past half millennium. These ten chapters are bracketed by introductory and concluding chapters. The approach ranges from a consideration of the use of processed plants, such as tea, to the use of purified extracts, such as cocaine. Throughout, however, there is a stress on the complexity of the issues involved, as substances wander in and out of legality with time and place.

The other volume, *Drugs and Narcotics*

in *History*, complements many of the topics covered in the first. Two of the authors of this book write separate dustjacket blurbs for the first. Much the same agents are discussed, but the approach is historical rather than anthropological or sociological. One consequence is vagueness in the use of pharmacological terms. John Scarborough talks of "so-called 'addictive' substances", and compares the daily taking of salicylate for cardiac and vascular problems with an addiction. This is unfortunate on a number of levels, not least of which is that salicylate is inactive as a vascular agent and cannot be equated with aspirin (acetylsalicylate). Furthermore, most regular users of aspirin are taking it to prevent vascular problems (that is, as a prophylactic) rather than to treat them.

As with the wine of the Christian mass, the cultural and social contexts are often more important than the psychoactive agent used. If the medium can be the message, then the context and package can be the 'reward'. Thus, the West Coast coffee 'ceremony' can involve decaffeinated coffee. Concern about health and postprandial functioning has led many to eschew the lunchtime cocktail for an expensive glass of attractively packaged water. Cults have arisen around the mystique of Evian and Perrier. The reason for this transference, as pointed out by Andrew Sherratt in his introduction, derives from the fact that "the substances themselves are often taken as a metaphor for a variety of social relationships". A cup of tea can be emblematic of the highest values of civilization,



SMELLS of splendour — this detail from Tutankhamun's royal golden throne shows his wife, Queen Ankhesenamun, offering him a vase of scented ointment. The picture is reproduced in *Egypt: Splendours of an Ancient Civilization* by Alberto Siliotti, which presents in large format some of the finest photographs taken of Egypt's monuments and treasures. The accompanying text gives an overview of 5,000 years of Egyptian history from the first pharaohs to the modern Arabic republic. Site plans, cross-section diagrams, a double fold-out map of the course of the Nile and an illustrated glossary make for a truly lavish and informative volume. Thames and Hudson, £29.95.

whether taken in an English drawing room or a Japanese tea house. Coca-Cola can recreate one's past, when "to be young was very heaven". Wine can be the blood of Christ, or southern sunshine. Indeed, it has been said that wine in the Mediterranean is practically synonymous with civilization. An Italian acquaintance who was advised to stop imbibing for health reasons sadly remarked to me: "I suppose it's possible to live without wine, but what's the point?" The social significance of "wine that maketh glad the heart of man" is exemplified by the symposium, so important to twentieth-century scientists. This derives from the Greek *symposion*, or drinking party. Like the ancient Greeks, modern scientists also find that the symposium can give rise to philosophy, politics and other extrapharmacological activities.

Discussions in the first volume make clear that the wandering legalities of psychoactive agents, licit in some societies, illicit in others, have little to do with pharmacology, whether they concern the banning of alcohol in some Muslim countries or the banning of cocaine in the United Kingdom and the United States. As Rudi Matthee suggests in the second volume, illegality has more to do with social factors. Muslim religious leaders originally objected to the coffee house because it competed with the mosque. In eighteenth-century England, an association was made between coffee houses and sedition, because of the free political discussion that occurred in them. But the ability to raise money competitively antagonizes morality. Taxation replaces prohibition in many societies.

Most psychoactive agents — coffee, tea, heroin, cocaine — originate from underdeveloped countries. After oil, coffee is the second most important export from the developing world to the first, employing some 20 million people. The combined trade in both legal and illegal psychoactive substances probably far outweighs the value of oil from developing countries. The amplification in value between producer and consumer — between the poppy or coca grower and the Manhattan mainliner or Angeleno snorter — makes the economic consequences of drug use more malign than the pharmacological consequences. The economies of producing countries are distorted, and food crops for local consumption are displaced by drug crops for foreign markets. The high retail cost of illegal agents forces users to crime and prostitution to raise the money needed. The latter does not apply to coffee and tea, of course, where free-market economics match supply and demand. It is no wonder that, as Ann Dally points out, the people who would least like to see drugs decriminalized are those who make their money from them.

Both books have the same attitude towards the criminalization of drugs, suggesting that drugs are made dangerous by

myth, politics, illegality and social factors rather than by their psychoactive or pharmacological properties. *Drugs and Narcotics in History* contains a polemical and spirited attack by Dally on the establishment approach to drug use in Britain. She accuses the government of using the drug issue to divert attention from the real issues. One can certainly sympathize with her attitude from the perspective of the United States, when one considers the rhetoric and resources applied to the drug 'problem' alongside the neglect of other important social issues, such as prenatal and antenatal health care.

Consuming Habits is a learned and thoughtful book, one that will stimulate readers from a variety of professional backgrounds. *Drugs and Narcotics in History* is both more technical and less integrated, with chapters on drug regulation in Victorian England and changes in alcohol use among Arizona Indians seeming to reflect availability of authors rather than the exigencies of the subject. Both books raise and discuss many more issues than can be mentioned in a short review. If you can afford only one book, buy the first. Together, however, they make a good pair. □

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Gratefully dead

John L. Casti

Metapatterns: Across Space, Time, and Mind. By Tyler Volk. Columbia University Press: 1995. Pp. 296. \$31.50, £21.50.

At least since the appearance of Fritjof Capra's book *The Tao of Physics* in the late 1960s, the popular-science literature has seen a steady stream of volumes purporting to show how science is simply part of a grand, unified scheme dovetailing with art, music, literature and just about every other human activity, not to mention the affairs of nature. Whether these books are offering a supposed bridge between elementary particles and Eastern religions or putative connections between the mysteries of quantum theory and human consciousness, they are all products of an intellectual age that one can only hope died with Jerry Garcia, lead guitarist of the rock band "The Grateful Dead", an age dedicated to the dubious hypothesis that everything and everyone is 'meaningfully' connected to everything else by deep, profound — yet never quite articulated — patterns of unity and wholeness. *Metapatterns* is a worthy addition to the list of volumes of this ilk.

Tyler Volk offers up a veritable cornucopia of what he calls "patterns of patterns" or "metapatterns". These range

from simple geometrical objects such as spheres and tubes to temporal structures such as cycles and even more metaphorical categories involving layers, centres and binaries. A Herculean effort is then expended in trying to suggest, if not show, that these structures permeate virtually everything we see and do. For example, the dome of the Roman Pantheon, the breasts of Hindu goddesses, and a stone finial on a gate post in Slovenia are related as part of some final theory of the Universe in which spheres play a leading role. In a similar vein, we find connections suggested linking the hierarchical structures of the US National Science Foundation organizational chart, the Islamic view of the heavens and the stems of berry plants. And so it goes, onward and upward through nearly 300 pages, complete with a rather extensive collection of photographic montages created presumably to portray these myriad connections as they are seen in the author's mind. What can one possibly make of such a work?

One way to evaluate this kind of intellectual outpouring of the soul is to ask if it either entertains or enlightens (or, possibly, both). Perhaps surprisingly, by the first criterion the book does actually provide the reader with a certain kind of entertainment. This is entertainment of the type one gets by the random perusal of an encyclopaedia or dictionary. Odd facts, curious (pseudo)connections and offbeat items of arcana are all woven together with a readable, almost chatty, conversational style that makes the story worth reading. That's the good news.

The bad news is that *Metapatterns* fails to shed light on even one major scientific question of our day. How did life start on Earth? Can machines think? How do children acquire spoken language? What is the nature of quantum reality? Are human behavioural patterns genetically determined? Just add your favourite 'big question' to this list.

After digesting Volk's account of metapatterns, I'd bet my next month's salary that you will not have gained a single iota of insight into the question from having read this book. So, even though the book must contain several hundred allusions to connections between domains not normally seen to be connected, I failed to find a single such connection made sufficiently explicit that it might actually offer the promise of illuminating even one important scientific issue.

And therein lies the weakness of all these 1960s-style books trying to bridge the Grand-Canyon-sized gap between science and the rest of intellectual life. Vague metaphors, spurious linkages and fuzzy allusions are just not enough. For notions such as centres, calendars, binaries and all the rest of Volk's menagerie to make contact with real scientific questions, these metapatterns need to be made

explicit and detailed enough in their connections with actual questions that one can see a reason why seemingly disparate areas might be related at a deeper level. Sometimes this can actually be done, as for example when one invokes the mathematical fact that the sphere encloses the largest possible volume for a given surface area. This fact then enables one to explain why spheres are so prevalent in both natural and human structures. But this kind of argument is sadly lacking for all the metaphors described here. So in just the same way that Jerry Garcia and his band of merry men failed to engage any of the real issues of their time, Volk has written a book that entertains lightly but ultimately fails to change one's view of the world in the slightest. □

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Missing history

Malcolm W. Browne

Triumph of Discovery: A Chronicle of Great Adventures in Science. Holt/Helicon: 1995. Pp. 254. \$40, £20.

WHAT could be more welcome to the serious science fan than a selection of gems from *Scientific American* spanning its 150-year publishing history? A lively and intellectually challenging intermediate between the professional journals and the popular press, *Scientific American* occupies an important publishing niche. Many of the greatest names in science have been pleased to contribute to the magazine over the years, and it is relished each month by both scientists and outsiders.

Unhappily, this handsome looking volume has little if anything to do with *Scientific American*, aside from its title. Some giants of present-day science (including several Nobel prizewinners) are represented in the book's grab-bag of 48 science nuggets, but each essay is limited to just a few hundred words, and in most cases the contributions are scarcely more illuminating than summary entries in an encyclopaedia. A reader who expects a level of sophistication comparable to that offered by *Scientific American* itself (or, indeed, by *Nature*) will be disappointed.

In its attempt to itemize great achievements of the past century-and-a-half, the book falls short. Every few pages the publisher has inserted thumbnail chronologies that note the years in which significant scientific, technological and historical events occurred, but the net used to winnow out accomplishments worthy of mention seems to have had many holes. Even some benchmarks in which *Scientific American* was

directly involved are ignored.

Although several of the book's articles discuss advances in weaponry, for example, the editor fails to mention that *Scientific American* served as a patent agency during its early years, and one of its clients during the American Civil War was Richard Jordan Gatling, inventor of the Gatling gun — a device that helped to change history.

Each of the essays was evidently edited independently of all the others, and in some cases overlapping treatments hint at great debates without explaining what these debates are about.

For instance, Peter H. Raven, director of the Missouri Botanical Garden, writes that a major extinction took place at the end of the Mesozoic era, which killed off two-thirds of all land animals and all of the dinosaurs, and "was probably linked with the impact of a huge asteroid". But elsewhere in the book, Kevin Padian, curator of the Museum of Paleontology at the University of California, Berkeley, writes that although there probably was a huge asteroid, "this catastrophe did not substantially affect the dinosaurs, because (except for the birds) as far as our records show, they were already extinct, and in fact their diversity had been dwindling for years". Readers familiar with the Cretaceous-Tertiary extinction debate will understand the bases of this contradiction, but for schoolchildren for whom this book seems to have been intended, some explanation would have been helpful.

The criteria by which contributors were selected lack balance. A case in point is the book's treatment of efforts to develop hydrogen fusion as an energy source. Ronald C. Davidson, director of the Princeton Plasma Physics Laboratory — the proprietor of the most powerful tokamak fusion machine in the world — provides an excellent summary of the state of magnetic-confinement fusion experiments, notably those at his own laboratory. But *Triumph of Discovery* does not allude to the probability that Congress will radically slash funding of the Princeton laboratory, or that a competing fusion system under development at Lawrence Livermore Laboratory, inertial confinement, may fare better than the Princeton tokamak after available funds are reapportioned.

Despite the confining format it imposes on its contributors, the book contains some worthy entries by scientists whose prose is as striking as their research. Among them are Thomas R. Cech discussing his prizewinning research on catalytic RNA; Virginia Trimble, who walks us through some of the great astrophysical discoveries based on spectral analyses; Anne H. and Paul R. Ehrlich, who recapitulate their plea for reining in catastrophic population growth; Steven Weinberg, who discusses the physicist's quest for underlying unity in nature; and Niles Eldredge, who outlines the evolutionary theory of punctuated

equilibrium, of which he and Stephen Jay Gould were the authors.

Some irksome typographical errors reflecting sloppy editing blemish the text. For instance, the printer seemed unable to cope with superscripted exponents, and a good survey by Michael S. Turner on current theories of cosmology says that the hypothesized inflationary period of the Universe lasted "10 to 32 seconds" where the author surely must have written "10⁻³² seconds".

Triumph of Discovery has its points, but it should have been a lot better. □

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Children's Books

Nature plans to publish on 16 November a supplement in which children's books and software and software will be reviewed. The supplement will cover science books and software for children of all age groups.

Publishers are invited to send suitable material for consideration, taking note of the following criteria:

■ Only books and software issued in 1995 will be considered;

■ Books and software dealing with any aspect of science, technology, medicine or natural history are eligible (including encyclopaedias, dictionaries and games), although school curricula texts are excluded;

■ The main language used must be English;

■ If possible, cross-platform software (both Macintosh and PC) should be provided.

Deadline for submission is 22 September.

Publications for review should be sent, together with details of price and availability, to Peter Tallack, Book Review Editor, *Nature*, 4 Crinan Street, London N1 9XW, UK (tel: +44 (0)171 843 4567; fax: +44 (0)171 843 4596/7; e-mail: p.tallack@nature.com).

New in paperback

Evolution Extended: Biological Debates on the Meaning of Life edited by Connie Barlow. MIT Press, £17.95. This anthology

of selections from the writings of biologists and philosophers offers a "valuable introduction to many old controversies and a few new ones", said Christopher Wills in *Nature* last year.

All About Arthritis: Past, Present, Future by Derrick Brewerton. Harvard University Press, £9.95, \$15.95. [The author] skilfully guides us through the complexities of bacteria, viruses, and host defence; the nervous system; the structure and function of joints; the mosaic of factors involved in arthritis; and the prospects for new forms of therapy. He is particularly elegant when dealing with the complex (and controversial) influence of personality and emotion", said David Isenberg in *Nature* in 1992.

An everyman's epidemiology

L. J. Kinlen

Investigating Disease Patterns: The Science of Epidemiology. By Paul Stolley and Tamar Lasky. *W. H. Freeman/Scientific American Library*: 1995. Pp. 242. \$32.95, £19.95.

THIS book aims to introduce nonspecialists to the science of epidemiology. Its practitioners study differences in disease incidence between groups of people in order to identify causes and quantify risks, but always with prevention in mind. These objectives are pursued by simple methods, particularly by comparing affected patients and healthy controls in respect of their exposure to a suspected cause and by following up healthy people exposed to the possible cause to find if they do in fact have a high incidence of the disease. The latter (cohort) type of investigation is time-consuming, but sometimes it reveals more widespread effects of a suspect cause than had originally been envisaged — as with smoking and coronary heart disease. Aided by statistical procedures and a rationale for distinguishing causal from indirect associations, epidemiology has made extensive contributions. In the field of cancer alone, it has revealed the role of tobacco, asbestos, hepatitis-B infection, sunlight, ionizing radiation and several chemicals.

The subject has come far from its origins when epidemics of infectious diseases were the dominating concern and when John Snow visited the homes of cholera victims in London in the 1850s to establish the source of their water. Cholera mortality was found to be almost ten times greater in households served by the company that drew its water from the lower Thames than in homes (often in the same street) supplied from higher up the river by a rival company, and it was this finding that enabled London to be protected from the disease. Epidemics are now considered to include increases of any disorder, even when protracted over several decades, such as lung cancer and coronary heart disease. But basically it is differences in the pattern of a disease that are the focus of interest, and because hardly any disorder occurs uniformly in all populations, effectively all are open to epidemiological investigation.

It is perhaps chastening to anyone tempted to look down on a subject that is largely (and necessarily) non-experimental that prevention is sometimes made possible even before the specific cause has been elucidated. London was protected from cholera before its bacillus had been identified; other examples include the

means of preventing pellagra before the discovery of nicotinic acid, and of preventing lung cancer before any specific chemicals (among thousands) in tobacco smoke were incriminated.

This book covers a wide field ranging from the hazards associated with maternal rubella and lead to the evaluation of screening procedure. There is a chapter on iatrogenic disorders (entitled "Medicines that backfire") covering the modern epidemics of fatal asthma from certain pressurized aerosols and of genital cancer in the adolescent daughters of women who had taken diethylstilboestrol in pregnancy. I found little to criticize except that the account of the classic work of Richard Doll and A. B. Hill reads as if they did not conclude that smoking actually causes lung cancer, whereas in fact they did. Not all epidemiologists would join the authors in claiming controlled trials of treatments for their subject. It also seems odd to find only variations in susceptibility mentioned, but not chance, in the discussion of why some individuals heavily exposed to a carcinogen should escape cancer.

Epidemiology represents the scientific foundation of public health and preventive medicine and the authors consider it to be an undervalued science. This attractively produced and richly illustrated volume will help many to appreciate its importance, scope and fascination. □

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Breathless niches

John Postgate

Ecology And Evolution in Anoxic Worlds. By Tom Fenchel and Bland J. Finlay. *Oxford University Press*: 1995. Pp. 276. £35, \$58 (hbk); £18.95, \$31 (pbk).

MICROBES have long been recognized as important agents of change during the evolution of the biosphere and as regulators of the environmental metastability that sustains life today. Yet for decades (to be unfair to a few dedicated scientists) microbial ecology remained largely a bland backwater of microbiology. Recently, however, the situation has changed dramatically, sparked as so often by the development of new research tools. The subject has become a fast-moving research area in which spectacular advances in microbiological knowledge have been made, some bearing on our understanding of early biological evolution.

Fenchel and Finlay, themselves distinguished contributors in this area, survey the physiological ecology of the anaerobic

microbes that inhabit the anoxic niches on our planet. These are numerous, ranging from aquatic sediments to animal intestines. Chapter 2 introduces the diversity of metabolic chemistries displayed by anaerobic bacteria (fermentation, phototrophy, acetogenesis, methanogenesis, sulphate reduction and so on) and provides an especially useful account of inter-bacterial syntrophy and the role of hydrogen cycling. Chapter 3 concentrates on the anaerobic protozoa (the only eukaryotic anaerobes), opening with some wise comments on their early evolution and going on to discuss the now numerous examples, some dependent on endo- or ecto-symbiotic prokaryotes. This chapter should be required reading for general microbiologists, many of whom will have encountered these fascinating creatures only as a footnote to rumen microbiology. Chapter 4 surveys the structures, spatial and sequential, of anaerobic microbial communities in various ecosystems. These chapters, complemented by a brief summary of the ecology of oxic-anoxic interfaces in chapter 5, are the meat of the book; all highly informative and up to date, except in their brief mention of nitrogen fixation; and the impression given on page 195 that microbial activity in sediments all but ceases below a depth of about 20 cm neglects the bacterial ecosystems that flourish 0.5 km deep in Pacific Ocean sediments (R. J. Parkes *et al.* *Nature* **371**, 410; 1994).

The ecology, then, is erudite. On evolution, for which the scene is set in chapter 1, some plausible scenarios are presented, especially concerning the history of eukaryotic organelles. Yet on the broader sweep of prokaryote evolution, the authors seem too ready to accept palaeochemical orthodoxy (for example that biologically significant free O₂ was present in the very early biosphere, and that free O₂ preceded SO₄ and NO₃, propositions that both disregard the oxidative effect of photolytic OH). And they could have been firmer about the message of promiscuous bacterial gene transfer. They rightly remind us that the new ribosomal RNA phylogeny is the phylogeny of rRNA genes, not of their hosts, but they do not follow the logic through: although certain physiological processes may be primitive or ancient, modern bacteria that display them are neither primitive nor ancient; and they are not necessarily, or even probably in some cases, linear descendants of the earliest forms possessing such physiologies.

But I carp. Comprehensive, clear, thought-provoking and occasionally witty, this book is a rewarding update for advanced students and researchers. □

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Determination of the Hubble constant from observations of Cepheid variables in the galaxy M96

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New Hubble Space Telescope observations of Cepheid variable stars in the nearby galaxy M96 give a distance to the host galaxy group, Leo I, of 11.6 ± 0.8 Mpc. This value, used in conjunction with several reliable secondary indicators of relative distance, constrains the distances to more remote galaxy clusters, and yields a value of the Hubble constant ($H_0 = 69 \pm 8 \text{ km s}^{-1} \text{ Mpc}^{-1}$) that is independent of the velocity of the Leo I group itself.

THE value of the Hubble constant, H_0 , has been the focus of one of the most colourful and heated debates in astrophysics. The ratio of expansion velocity to distance, H_0 is of fundamental importance for testing the standard Friedmann world models, which have formed the essential framework of cosmology for the past half century^{1,2}. Most published values fall in the range $40 < H_0 < 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, often with formal uncertainties of less than 10% (refs 3–5). The intense interest in this debate stems from the fact that a value for H_0 greater than $75 \text{ km s}^{-1} \text{ Mpc}^{-1}$ corresponds to an age for the Universe of < 8.7 Gyr in the favoured Einstein–de Sitter model. This would not only be in severe conflict with the inferred ages of the oldest globular clusters⁶ but would also be less than the estimated age of the galactic disk from nucleo-cosmochronology⁷ and the local white-dwarf cooling sequence⁸.

From observations made with the Hubble Space Telescope (HST), described here, we obtain a Cepheid distance to the galaxy M96 in the Leo I group of 11.6 ± 0.8 Mpc. We then use the ‘early-type’ galaxies (elliptical and lenticular) in Leo I to deduce a distance to the Coma cluster of 105 ± 11 Mpc. The velocity of Coma in our microwave background rest frame is taken as $7,200 \pm 300 \text{ km s}^{-1}$, where the uncertainty is intended to include allowance for the possible peculiar velocity of Coma itself⁹. Hence we find a Hubble constant of $H_0 = 69 \pm 8 \text{ km s}^{-1} \text{ Mpc}^{-1}$, which corresponds to an age of the Universe in the standard Einstein–de Sitter ($\Omega = 1$, $\Lambda = 0$) cosmology of 9.5 ± 1.1 Gyr. Estimates of the ages of the oldest globular clusters are generally between 12 and 17 Gyr (ref. 6). Only for the lowest values in this range is our result even marginally consistent with the Einstein–de Sitter model.

HST and the distance scale

The ability of HST to resolve and monitor Cepheid variables at greater distances than previously possible represents a major opportunity to improve the local distance scale. Unfortunately, even at distances accessible to HST, galaxy ‘peculiar’ motions are significant compared with Hubble expansion velocities⁹. It is therefore necessary to calibrate secondary distance indicators to extend the distance ladder to regimes where corrections for peculiar velocities are small.

Some secondary indicators can be calibrated directly by using the spiral galaxies in which Cepheids are found. In particular, significant amounts of HST time are being devoted to calibrating the absolute magnitude of type Ia supernovae¹⁰ and determining the zero-point of the infrared Tully–Fisher relation¹¹. However, even with these advances, long-standing disputes over the appli-

cation of these indicators may delay consensus. The disputes centre on (1) the intrinsic scatter in the luminosity of type Ia supernovae^{12,13} and the reliability of historic photometry for the calibrating supernovae¹⁴, and (2) the intrinsic scatter of the Tully–Fisher relation^{15,16}.

A third and complementary route is to consider instead the various secondary indicators developed for ‘early-type’ galaxies. These galaxies have the important advantage over spirals that they congregate in the cores of groups and clusters, allowing many galaxies to be usefully averaged in the process of distance determination. Several of these methods have been shown to give reasonably consistent relative distances^{17,18}, but the lack of nearby normal giant elliptical galaxies makes calibration difficult. In the past, attempts at calibration have been made by using the bulges of nearby spirals, notably the Milky Way, M31 and M81. Unfortunately there is an inevitable ambiguity when calibrating a method in a galaxy of a different type from that in which it is subsequently employed. Differences in stellar populations and the presence of disk stars and dust could introduce systematic errors.

With the HST it is now possible to pursue the alternative approach of calibrating these relations by measuring Cepheid distances to spirals in groups and clusters that also contain ellipticals. The worry here is that neighbours in projection on the sky may in fact be separated along the line of sight. This problem is illustrated by the recent work on the galaxy M100 (ref. 19). The excellent HST photometry gives a very reliable distance to M100 itself; the error in determining H_0 is dominated by the uncertainty in the relative position of M100 compared with the Virgo cluster core. The Virgo cluster is particularly problematic because of its known complexity of structure²⁰, and the evidence that the late-type members are significantly extended along the line of sight^{21–23}. There are other, smaller, galaxy groups within the reach of HST, but there is comparable uncertainty in the physical association of the spiral members^{24–26}.

The galaxy M96 (=NGC3368, $\alpha_{2000} = 10 \text{ h } 46 \text{ min } 45.2 \text{ s}$, $\delta_{2000} = 11^\circ 49' 16''$) offers the chance to break this impasse. It resides in a moderately compact group that has been variously named the M96 or Leo I group. It is the nearest group to contain a good mix of early- and late-type galaxies (see ref. 24 for a chart). The three main early-type galaxies are found to be at the same distance by the surface brightness fluctuation (SBF) and planetary nebula luminosity function (PNLF) methods¹⁸. The group contains a unique ring of intergalactic neutral hydrogen gas²⁷ which, from its kinematics, is thought to be orbiting the central two early-type galaxies M105 and NGC3384. There is

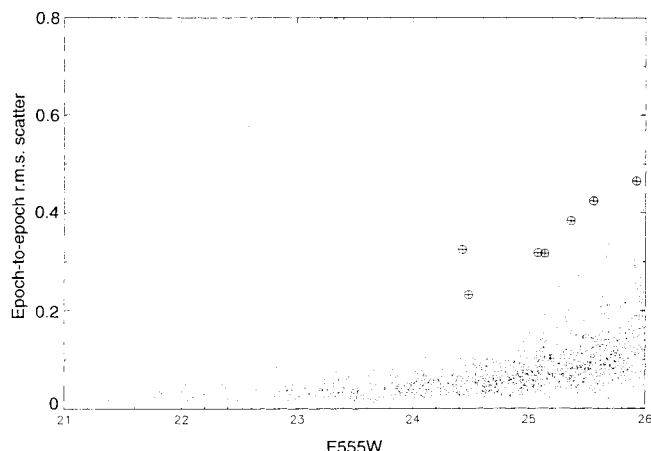


FIG. 1 A plot of instrument V magnitude (filter F555W) against the epoch-to-epoch r.m.s. scatter. Each epoch is split into two exposures to help identify cosmic rays, and the magnitude at each epoch is obtained in a 0.3-arcsec aperture. We selected an outer field in M96, and the level of crowding at this depth is moderate. This, combined with the poor WFPC-2 sampling, justifies the use of aperture photometry, particularly while the temporal and spatial variations of the WFPC-2 point spread function (psf) are not well understood³⁰. The median r.m.s. scatter at $V=26$ mag is ~ 0.18 mag, similar to that obtained for M100 (ref. 19), although this varies with position on the frame owing to the changing background level across the field. The 8 Cepheids are marked and are clearly separated from the main locus of stars. The brightest Cepheid, marked with an uncircled cross, was observed at only 8 epochs and its period and magnitude are correspondingly uncertain. Some of the other stars with high scatter may be other types of variable, although a few outliers are expected from noise and occasional contamination of stars by 'hot pixels' or cosmic rays. The Cepheids can also be identified by the shape of their light curves.

a tail of gas extending from the ring towards M96, which is conventionally interpreted as evidence of an interaction²⁴. M96 is already very close in both projection and velocity to the central pair, so this apparent physical connection gives us some confidence that it is at the same distance as the early-types. It is therefore an ideal location to tie together the Cepheid distance scale and the early-type galaxy scale.

Cepheid observations

We obtained HST wide-field and planetary camera-2 (WFPC-2, ref. 28) images of M96 at 13 epochs spaced over 7 months. A 40-min V -band (filter F555W) integration was acquired at each epoch from which we obtained the periods and average V magnitudes of the variables. At three of the epochs we also obtained 50-min I -band (filter F814W) images which were used

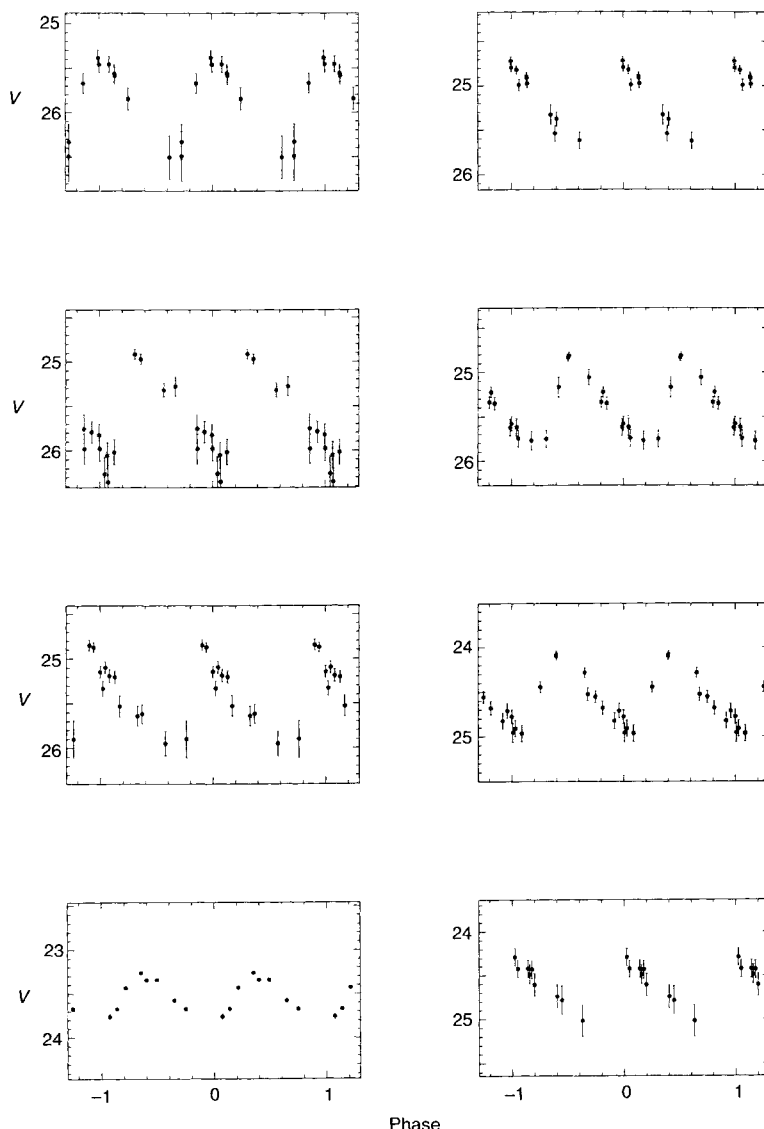


FIG. 2 Light curves of the 8 Cepheids folded on the best periods. The amplitudes of variation are comparable with those seen in studies of nearer galaxies (for example ref. 11), indicating that there is unlikely to be a problem with field stars overlapping the Cepheid images. Approximate periods were determined by a number of automated methods, all of which agreed well with each other. Ultimately the visual fit was considered most reliable although in no case did this make a significant difference. The well-known 'saw-tooth' signature of classical Cepheids is evident. The telescope pointing was shifting by a small amount between most epochs and, in particular, the final three epochs were obtained at a position angle $\sim 180^\circ$ from the others. Thus some stars were not imaged at all epochs. In addition, the large number of cosmic rays and 'hot pixels' on many of the images meant that, although each epoch was split into two exposures, occasionally a star was 'hit' in both, and hence no magnitude was found for that epoch. All the Cepheids used in fitting the P - L relations had good photometry for at least 9 epochs. Only for the longest period variable (bottom left panel) is the sampling too poor to be sure of the magnitude or period (there is an alias at ~ 100 days).

to constrain the colours and hence account for the extinction by dust. The epochs were originally scheduled to allow good sampling of periods in the range 10 to 65 days. A large gap of 120 days was left between the 10th and 11th epochs to ensure that the periods for most of the variables were quite precise and free of aliases. Our strategy was altered slightly by an unscheduled HST shutdown that occurred early in our programme, resulting in the rescheduling of one epoch.

The full procedure for obtaining photometry will be described in more detail elsewhere (N.R.T. *et al.*, manuscript in preparation). Briefly, accurate mappings were obtained between the coordinate systems of each individual image, using about 100 bright stars. A master co-added image was then created and

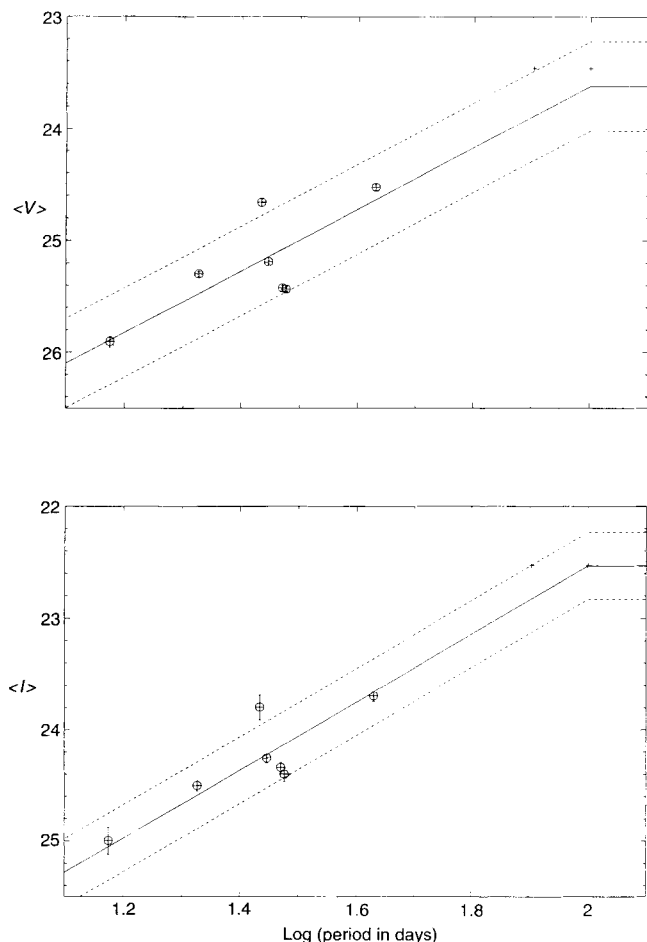


FIG. 3 Here we fit the Cepheid P - L relations of Madore & Freedman³² to both the V and I data. For some time the calibrations of the Cepheid variable (P - L) relations have been in good agreement, as shown by the various Galactic calibrations³⁹, and the several independent distances to the Magellanic Clouds, which are generally consistent with their Cepheid distances^{4,40}. The level of agreement is illustrated by the common use of the Madore & Freedman³² P - L relations by advocates of both low¹⁰ and high³⁸ values of H_0 . The average Cepheid magnitudes are defined as the magnitude at mean intensity. The slopes are fixed to be 2.76 in V and 3.06 in I from the Magellanic-Cloud Cepheids³² so there is only one free parameter in each case. The approximate envelopes of the Magellanic-Cloud Cepheids are also plotted. Only 7 Cepheids are included in the fit because the brightest variable is of uncertain period (it is plotted with both 80 and 100 days) and is close to the point where the P - L relations become nonlinear and not well understood. The error bars are derived only from photon-counting statistics and therefore do not account for errors in fitting the light curves and errors due to crowding; however, they provide reasonable weights for the P - L fits. The effective magnitude limit at $V=26$ is marked; we expect any bias due to incompleteness to be small.

TABLE 1 A complete error budget used in deriving the distance to Coma

	Source of uncertainty	R.m.s. magnitude error
[A]	Adopted V and I zero-point error	0.03
[B]	Spread of reddening corrected distance moduli from 7 Cepheids	0.21
[C]	Uncertainty in LMC to Leo modulus ($2.45 \times [A]$, $1.45 \times [A]$, $[B]/\sqrt{7}$)	0.12
[D]	Uncertainty in LMC distance modulus ³² (i.e. the Cepheid calibration)	0.10
[E]	Allowance for possible metallicity effect on Cepheid P - L relation	0.05
[F]	Uncertainty in M96 distance modulus ([C], [D], [E])	0.16
[G]	Estimated r.m.s. depth of Leo I group (see text)	0.04
[H]	Uncertainty in Leo I group centroid ([F], [G])	0.17
[I]	Adopted Leo I to Virgo uncertainty from Fig. 4	0.15
[J]	Adopted Virgo to Coma uncertainty (see text)	0.10
[K]	Leo I to Coma: route via Virgo cluster ([I], [J])	0.18
[L]	Leo I to Coma: route via $D_n - \sigma$ relation	0.34
[M]	Combined Leo to Coma from weighted mean of [K] and [L]	0.16
[N]	Resultant error in Coma modulus ([H], [M])	0.23

Figures in parenthesis are added in quadrature to obtain the result. Numbers are generally rounded to two significant figures. Note that our step [B], in which we find the spread in the 7 independent estimates of $\mu_0 = 2.45\mu_{A_V} - 1.45\mu_{A_I}$, automatically accounts for the correlation between the V and I residuals from the P - L relations. The main difference between our budget and that of Freedman *et al.*¹⁹ for M100 is in the allowance made for the depth of the group. The strength of our result for H_0 depends on the validity of the arguments made for M96's being closely associated with the Leo I ellipticals. Note also that we are using the most recent WFPC-2 calibration by J. Holtzman *et al.* (preprint), which is more precise than the provisional calibration available when the M100 result was published. This largely accounts for our improved precision in obtaining the distance to M96 itself. The reddening relation $A_V = 2.45E_{V-I}$ also comes from Holtzman *et al.*

used to obtain a coordinate list of stars down to a faint magnitude limit ($V \approx 26.2$ mag). This list was then transformed back to create a coordinate list appropriate to each frame. Aperture photometry was performed with the DAOPHOT-2 PHOT routine as implemented in IRAF²⁹. Aperture corrections were determined from bright stars on the frames themselves and are accurate to ~ 0.03 mag (ref. 30). We adopted the zero-points and colour terms from the WFPC-2 photometric calibration report (J. Holtzman *et al.*, preprint). Given that uncertainties still exist in the various corrections applied to the data, we adopt an error of 0.03 mag on the zero-point calibration of each band.

Several algorithms were employed to identify variables (one discriminator is shown in Fig. 1). All candidate variables were tested for periodicity with a Lafler-Kinman string-length statistic³¹. The Cepheid variables were selected from these on the basis of visual inspection of the light-curve shape and are clearly located well above most of the stars in Fig. 1. The light curves for the Cepheids are shown in Fig. 2. The brightest variable in our sample probably is a Cepheid, but was observed at only 8 epochs and there is a consequent uncertainty in both its mean magnitude and period (both ~ 80 or ~ 100 days are consistent with the data). In any case, it is in the regime ($\log_{10}(P) > 1.8$, where the period (P) is in days) that was excluded in the P - L calibration³² because Cepheids with $\log_{10}(P) > 2$ cease to lie on a linear period-luminosity (P - L) relation. We therefore omit this variable from the sample and are left with 7 Cepheids brighter than $V=26$ mag.

The P - L relations for the Cepheids are shown in Fig. 3 where we have fixed the slope of the relations and fitted only for the zero-point³². This gives $\mu_{A_V} = 30.51 \pm 0.12$ mag and $\mu_{A_I} = 30.42 \pm 0.09$ mag, where these uncertainties come from the

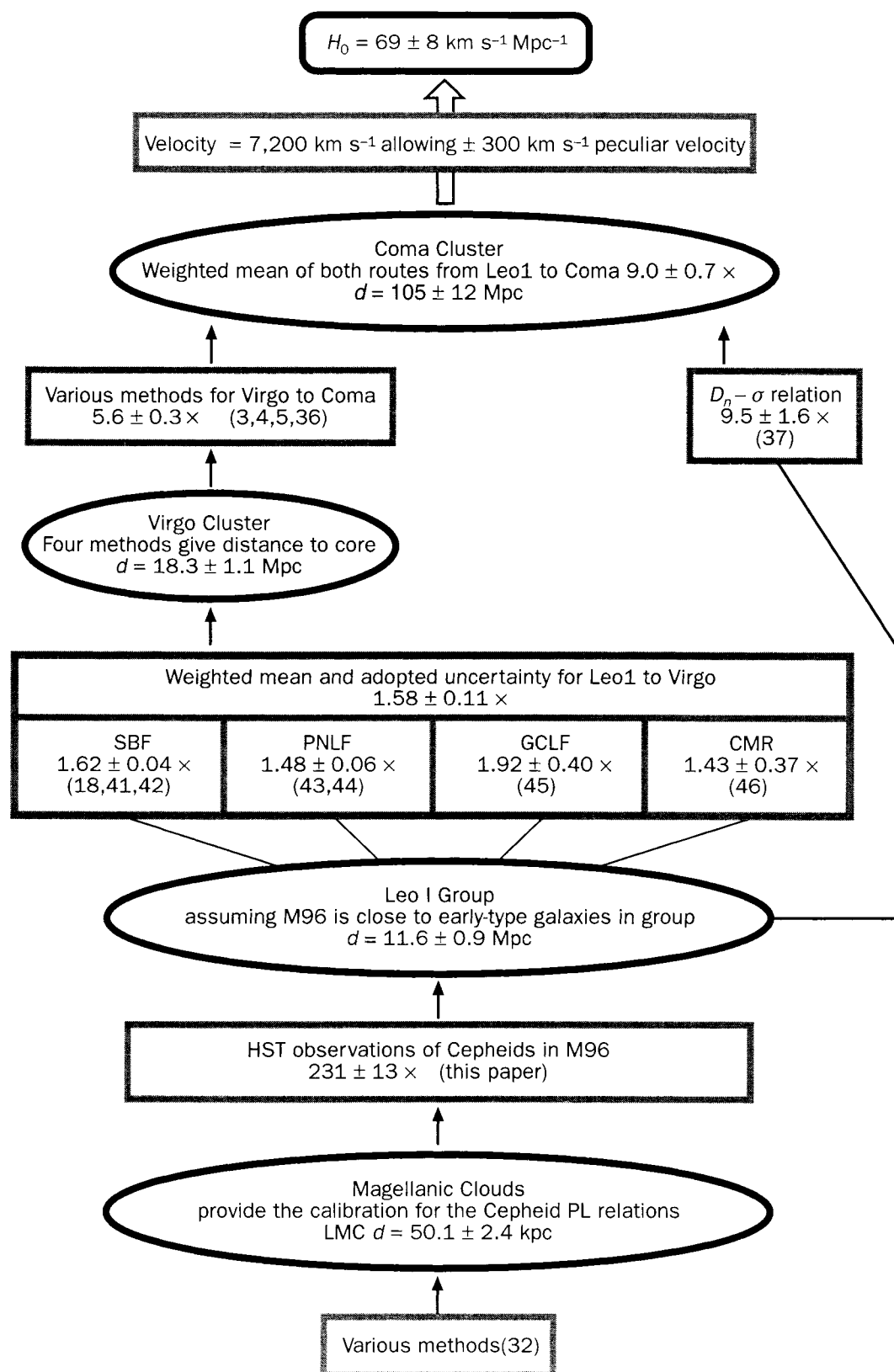


FIG. 4 This figure shows all the steps involved in deriving H_0 . Each box represents a distance ratio as inferred from data given in the papers referenced in parentheses. We start with the standard Cepheid $P-L$ relations obtained in the Magellanic Clouds³² and step via Leo I to the Coma cluster. The Coma cluster is assumed to be sufficiently remote that the corrections to its velocity for peculiar motions are small⁹. The two routes from Leo I to Coma rely primarily on various early-type galaxy-distance indicators, namely the SBF, PNLf, globular cluster luminosity function (GCLF), colour-magnitude relation (CMR) and $D_n - \sigma$ methods. Note that the $D_n - \sigma$ relation gives $d(\text{Virgo})/d(\text{Leo}) = 1.71 \pm 0.30$ (ref. 37), but this is omitted in the route via Virgo so that the direct Leo I to Coma route is kept as independent as possible. Although the formal error on the weighted mean in the Leo I to Virgo step is only $\sim 2\%$ we have allowed a larger uncertainty to account for possible systematic effects. As the early-type indicators are fairly consistent with each other, the value of H_0 we derive is reasonably insensitive to the choice of weighting.

observed dispersion around the $P-L$ relations. The difference between the two apparent moduli is interpreted as evidence of reddening due to dust, corresponding to a mean total extinction of $A_V = 0.19 \pm 0.11$ mag, where the error includes the uncertainty in photometric calibration. For comparison the foreground extinction to M96 from dust in the Milky Way is estimated to be only $A_V \approx 0.05$ mag (converted from $A_B = 0.06 \pm 0.06$ mag;

ref. 33). We note that although there should be a small incompleteness bias due to the magnitude limit of our current sample at $V=26$ mag, it could not be large given that our brighter Cepheids already populate the full envelope of the $P-L$ relation as estimated from the Magellanic Clouds.

The calibration of the $P-L$ relations, used above, is based on observations of Cepheids in the Magellanic Clouds³² and

assumes a distance modulus for the Large Magellanic Cloud (LMC) of $\mu_0 = 18.5 \pm 0.1$ mag and reddening $E(B - V) = 0.1$ mag. Combining the total internal error with the uncertainties in the photometric calibration gives $\mu_0(\text{Leo}) - \mu_0(\text{LMC}) = 11.82 \pm 0.12$ mag. Hence we obtain a true distance modulus for M96 itself of $\mu_0 = 30.32 \pm 0.16$ mag, which corresponds to a distance of 11.6 ± 0.8 Mpc. Any effect of differences in metal abundance between the LMC and our M96 field is expected to be small for $V - I$ colours³⁴, and we have included an uncertainty of 0.05 mag to allow for this.

The Leo I group is $\sim 6^\circ$ across, although most 'high-confidence' members are in a $2.5^\circ \times 1.5^\circ$ region. If we assume roughly spherical symmetry, this would imply an r.m.s. depth of $\sim 2\%$ of the distance to the group. In particular M96, given its apparent interaction with the H I ring surrounding the central galaxies, is probably even closer to the centroid. Therefore the depth of the group is likely to be negligible compared with the uncertainty in the distance. A summary of the error budget for our H_0 estimate is given in Table 1.

From Leo I to the Coma cluster

The Leo I group is relatively nearby and so its Hubble recession velocity is made uncertain by local peculiar motions⁹. We therefore consider two routes to step from the Leo I group to the more distant Coma cluster, whose peculiar velocity is small compared with its Hubble velocity (Fig. 4). First, we use the Virgo cluster as a stepping stone by adopting the Leo I to Virgo distance ratio obtained from the weighted mean of a variety of distance indicators. These are summarized in Fig. 4 and give $\mu_0(\text{Virgo}) - \mu_0(\text{Leo I}) = 0.99 \pm 0.15$ mag. The indicators with the highest claimed precision, and hence greatest weight, are the I -band SBF and the PNLf methods. The disagreement between these two methods, although small, seems to be significant given the quoted errors. There are reasons to suspect that the problem could lie with the PNLf method, which is reaching its limit at Virgo cluster distances and may also have a dependence on parent galaxy luminosity^{18,35}. However, our adopted uncertainty of 0.15 mag, which is more than three times the formal error, encompasses both results at the 1σ level. Although we are determining H_0 at the Coma cluster, we note in passing that the above implies a Virgo distance of 18.3 ± 2.0 Mpc, which is intermediate between the traditional 'short scale' of 13–16 Mpc and the 'long scale' of 20–24 Mpc.

The published estimates for the relative Virgo–Coma distance are generally in good agreement. Rather than summarizing all the available measures of this ratio we refer the reader to recent reviews^{3,36}, which conclude with values for $\mu_0(\text{Coma}) -$

$\mu_0(\text{Virgo})$ of 3.80, 3.71, 3.69–3.74 and 3.80 mag respectively. We therefore adopt 3.75 ± 0.1 mag with, again, a reasonably conservative uncertainty. Combining this with the Leo–Virgo relative distance gives $\mu_0(\text{Coma}) - \mu_0(\text{Leo}) = 4.74 \pm 0.18$ mag.

A second route to Coma is to establish the zero-point of the $D_n - \sigma$ relation³⁷ (a relation between diameter and internal velocity dispersion for elliptical galaxies) in the Leo I group itself by using the two ellipticals M105 and NGC3377. This has a greater uncertainty owing to the intrinsic scatter around the $D_n - \sigma$ relation, but offers a check that does not make use of the somewhat newer SBF and PNLf methods. The relative distance between these two Leo I galaxies and the mean for the Coma cluster is $\mu_0(\text{Coma}) - \mu_0(\text{Leo}) = 4.90 \pm 0.34$ mag (ref. 37). Although the $D_n - \sigma$ relation is also among the techniques used to obtain the Virgo–Coma distance ratio, because the uncertainty is dominated by the small number of Leo I galaxies this route to Coma is largely independent of the route via Virgo. The two routes agree well within their respective errors. Taking a weighted average of these results and our Cepheid distance to M96 we obtain a distance modulus to Coma of 35.10 ± 0.23 mag or a distance of 105 ± 11 Mpc. Hence we obtain $H_0 = 69 \pm 8 \text{ km s}^{-1} \text{ Mpc}^{-1}$ as described above.

Future prospects

Our value of the Hubble constant is based on the standard Cepheid $P-L$ relations and the reasonable assumption of small corrections for peculiar velocity at Coma. The intermediate step relies on calibrating the early-type galaxy distance scale in Leo I and thus avoids some of the disputes that have contributed to the long-standing distance-scale controversy. The result still has an uncertainty consistent at the 2σ level with either the 'high' values of Pierce *et al.*³⁸ and Freedman *et al.*¹⁹, or the 'low' value of Sandage *et al.*¹⁰. The size of the uncertainty depends on the arguments for M96's being in close proximity to the Leo I early-type galaxies. Continuing HST observations of UGC5889 and M95 (by our group and the key-project team) should help to test whether there is any significant extension along the line of sight and to refine the mean distance to the group.

Otherwise the error estimate makes reasonable allowance for many possible sources of external error and is not dominated by any single uncertainty. Further measurements with the various early-type galaxy distance indicators are required to improve the relative Leo I–Coma distances and also to establish whether there is a significant discrepancy between the SBF and PNLf methods. With these improvements, HST distances to Leo I group galaxies offer the prospect of a new and relatively secure route to determining H_0 . $\square$

Received 6 February; accepted 1 August 1995.

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ACKNOWLEDGEMENTS. We thank M. Irwin for obtaining a ground-based image of M96 that was useful in selecting the HST field centre; the staff at STScI, especially D. van Orsow, for assistance in planning and scheduling the HST observations; to J. Holtzman for clarification of WFPC-2 calibration; to D. Lynden-Bell for useful discussions; and M. Livio for a critical reading of the manuscript. This work was based on observations with the NASA/ESA Hubble Space Telescope, obtained at the Space Telescope Science Institute, which is operated by the Association of Universities for Research in Astronomy. H.C.F. acknowledges a Hubble fellowship.

Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor

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The crystal structure of the tandem SH2 domains of human ZAP-70 in complex with a peptide derived from the ζ -subunit of the T-cell receptor reveals an unanticipated interaction between the two domains. A coiled coil of α -helices connects the two SH2 domains, producing an interface that constitutes one of the two critical phosphotyrosine binding sites. These and other unique features provide the molecular basis for highly selective association of ZAP-70 with the T-cell receptor.

T-CELL recognition of antigen-presenting cells initiates a cascade of intracellular processes that ultimately result in changes in gene expression, the production of secreted mediators, and cellular proliferation^{1,2}. This recognition is mediated by the T-cell antigen receptor (TCR), which consists of the antigen-binding α - and β -subunits, the CD3 complex of γ -, δ - and ϵ - chains, and the ζ -homodimer. With the exception of α and β , the intracellular portion of each subunit includes one to three amino-acid sequences that contain the motif YXX(L/I)X(₇₋₈)YXX(L/I), where X is variable³. After receptor stimulation, these immunoreceptor tyrosine activation motifs (ITAMs) become phosphorylated on tyrosine residues and, in this modified form, provide binding sites for downstream signalling proteins.

The TCR has no intrinsic protein tyrosine kinase (PTK) activity; however, members of both the Src family and the Syk/ZAP-70 family of PTKs are implicated in the functioning of antigen receptors⁴. Current evidence indicates that Src family kinases phosphorylate the ITAMs of the TCR⁴. Zeta-associated protein (ZAP)-70 then associates with the doubly phosphorylated ITAMs of the ζ - and CD3 ϵ -chains through its Src homology-2 (SH2) domains⁵, and is itself phosphorylated during early T-cell activation⁶. ZAP is a protein tyrosine kinase of relative molecular mass (M_r) 70K that exists exclusively in T cells and natural killer cells⁷ and is important for T-cell activation. Genetic alterations in the ZAP-70 gene that cause loss of expression of ZAP-70 in humans prevent antigen activation of CD4⁺ T cells, inhibit maturation of CD8⁺ T cells, and lead to severe combined immunodeficiencies^{8,9}. The binding of ZAP-70 to the TCR is believed to be essential for signal transduction, as peptides that block the association of ZAP-70 with the ζ -chain also inhibit T-cell signalling events¹⁰.

ZAP-70 consists of two SH2 domains that are connected by a 65-residue segment (the inter-SH2 region) and are followed by a second connecting region and a catalytic domain⁷. Interaction of ZAP-70 with the TCR requires that both of its SH2 domains are present and functional, and that both tyrosines within the ITAM are phosphorylated¹¹⁻¹³. SH2 domains play an integral role in intracellular signal transduction by mediating protein-protein interactions through specific recognition of

tyrosine-phosphorylated proteins^{14,15}. Selectivity by SH2 domains is dependent upon recognition of residues immediately carboxy-terminal to the phosphorylated tyrosine (pY + n). The three-dimensional structures of several isolated SH2 domains (both liganded and unliganded)¹⁶⁻²³, as well as one SH3-SH2 complex²⁴ and one SH3-SH2-SH3 protein²⁵, have been determined by X-ray crystallography or nuclear magnetic resonance.

Here we report the X-ray crystal structure of the tandem SH2 domains of human ZAP-70 (ZAP-NC) in complex with a doubly tyrosine-phosphorylated 19-meric peptide that is derived from the sequence of the first ITAM of the ζ -subunit (ζ_1) of the TCR at 1.9 Å resolution. The structure determination is summarized in Table 1 legend. The ZAP-NC: ζ_1 complex involves an extensive array of contacts between the peptide and both SH2 domains. The 65-residue inter-SH2 region exists as a coiled coil of α -helices and assists in the formation of an interface between the two SH2 domains. Within this interface, both SH2 domains contribute to the recognition of phosphotyrosine in the second pYXXL motif. This study provides structural insights into the Syk/ZAP family of PTKs and describes an intracellular component of the TCR.

General topology

The first 259 residues of ZAP-70 consist of two SH2 domains that are connected by a helical region. The overall fold is Y-shaped (Fig. 1b): the SH2 domains make up each upper branch, and the intervening 65 residues form the stem of the Y. The fragment of ZAP-70 used in the crystal structure determination terminates before the kinase domain; we therefore refer to the two SH2 regions as ZAP-N and ZAP-C, for ZAP N-terminal SH2 and ZAP C-terminal SH2, respectively. There is high structural similarity between each of the SH2 domains and those previously reported, such as p60^{v-src} (ref. 16) and p56^{lck} (ref. 19). Each of the individual ZAP SH2 domains possesses a central antiparallel β -sheet that is flanked by two α -helices. The inter-SH2 region begins as a β -strand that is a continuation of the central sheet of ZAP-N. This is followed by two antiparallel α -helices that form a coiled coil. The two SH2 domains are side by side, with an angle of $\sim 52^\circ$ between the central β -sheets.

ζ_1 is extended over both faces of the SH2 domains, straddles both central β -sheets, and makes extensive contacts with the protein surface. The binding orientation is head to tail, that is, the amino terminus of the peptide is in contact with the C-terminal SH2 domain. Binding of phosphorylated peptides to

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individual SH2 domains has been described as reminiscent of a 'socket and plug'²⁰, where the phosphotyrosine (pY) and the pY + 3 residues are the prongs. This general arrangement is also present for each pYXXL contact with ZAP-C and ZAP-N. The peptide segment that separates the two pYXXL motifs is largely in contact with ZAP-C. The C-terminal phosphotyrosine is bound in a pocket that is formed by contributions from both SH2 domains (Fig. 1a,c). The nomenclature defined previously¹⁹ for structural features of SH2 domains is used here for clarity (Fig. 2). Both BC loops of ZAP-NC are extended.

The inter-SH2 domain

The inter-SH2 spacer begins in a type II reverse turn followed by a long β -strand that forms an extension to the central β -sheet of ZAP-N. This segment is followed by a five-turn α -helix (designated helix C) that extends away from both SH2 domains, forming the stem of the overall Y shape of ZAP-NC. This helix is followed by a turn and a second α -helix (helix D) that curves around the axis formed by helix C. Helix D is distorted by two breaks and a short 3_{10} -helix. Helices C and D both make several hydrophobic contacts to ZAP-C. These antiparallel helices form a coiled coil, with direct contact between several hydrophobic residues forming its core. Several water-mediated hydrogen bonds exist between helix D and ZAP-C.

Binding of the complexed ζ_1 peptide

The bound conformation of ζ_1 is extended, although nearly one full α -helical turn exists between residues ζ Asn 8 and ζ Arg 12 (Fig. 1c). All residues of ζ_1 , except for ζ Gly 10, are in contact with ZAP-NC. The area of the peptide-protein interface is over 1,300 Å². Although this interface area is typical for protein-protein interactions, the nature of the contacts is different from those generally observed²⁶. For example, interfaces in antibody-antigen complexes and protease:protein-inhibitor complexes usually contain few bridging water molecules. The interaction of ZAP-NC with ζ_1 includes 21 bridging water molecules. Most

of the contacts in protein-protein interfaces are usually classified as hydrophobic. In contrast, half of the contacts between ZAP-NC and ζ_1 are due to direct hydrogen bonds. The total number of contacts observed is considerably larger than is observed for protein-protein structures of similar interfacial areas.

Binding of motif-1 (-pYNEL-)

The N-terminal pYXXL motif of ζ_1 is associated exclusively with ZAP-C. The first two residues of ζ_1 are largely involved in intrapeptide interactions. The single contact between ζ Leu 3 and ZAP-NC, a hydrogen bond between its main-chain carbonyl and Arg 170, is typical of the pY - 1 residue of peptides bound to SH2 domains.

The pocket for ζ pTyr 4 is formed by residues from helix A, strands B, C and D, and the BC loop. Hydrophobic contacts involve residues from β D, from which His 210, Tyr 211 and Leu 212 form one edge of the pTyr cavity. The side chain of Leu 212 is twisted away from the pTyr ring and is packed against Trp 131 from a symmetry-related molecule. This neighbouring Trp, which constitutes the only intermolecular crystal contact with any ζ_1 residue, is also in hydrophobic contact with ζ pTyr 4. Direct hydrogen-bonding contacts to the phosphate are made by only three residues (Fig. 3a). Arg 170 (α A) and Arg 190 (β B) interact through their terminal nitrogens. Arg 192 is the only residue in the region of the BC loop that is of sufficient length for direct hydrogen bonding to the phosphate group and interacts via its N ϵ . The BC loop is extended, so the pTyr binding region resembles a deep groove that continues toward the AA loop. Five water molecules with very strong density and low temperature factors exist in this region and are part of a large hydrogen-bonding network.

As is typical for complexes with SH2 domains¹⁹⁻²¹, the pY + 1 and pY + 2 residues are extended along the surface of the protein. The pocket that surrounds ζ Leu 7 (pY + 3) is very deep and is formed by residues from β D, the EF loop, helix B and the BG

TABLE 1 Statistics for data collection, phase determination and refinement

Data collection	Resolution (Å)	Reflections (N)	Completeness (%)	R_{sym} (%) [*]	R_c [†]	Figure of merit	Phasing power (20–2.8 Å) [‡]
TML	25–2.5	15,506	98.3	6.3			
SeMet TML	25–1.9	23,978	98.9	4.9	0.71	0.50	1.38
Refinement							
Model 272 residues, 113 water molecules, 1 lead, 3 selenium atoms							
Resolution	Reflections ($F > 2\sigma$)	R-value (%) [§]	Free R-value (%)	R.m.s. deviations			
				Bonds (Å)		Angles (°)	
10–1.9	23,697	20.9	25.5	0.006		1.58	

Details of peptide synthesis and the expression and purification of ZAP-NC will be published elsewhere. The peptide (NQLpYNELNLGRREEpYDVLVD) was synthesized by automated solid-phase synthesis. ZAP-NC (residues 1–259) was expressed as a glutathione S-transferase (GST) fusion protein and cleaved with thrombin. **Crystallization.** ZAP-NC complexed with doubly phosphorylated ζ_1 and treated with trimethyllead acetate (TML) was crystallized by vapour diffusion in hanging drops containing 13.5 mg ml⁻¹ complex and 10% PEG 4000, 50 mM sodium citrate, 100 mM ammonium acetate, 0.005% sodium azide and 20 mM dithiothreitol, pH 6.2, over reservoirs of 20% PEG 4000 and 20 mM dithiothreitol. The crystals are monoclinic ($P2_1$, $a = 50.11$, $b = 63.37$, $c = 54.00$ Å, $\beta = 114.44^\circ$) with one molecule per asymmetric unit. **Data collection.** Diffraction data were collected at room temperature with a Rigaku R-Axis II area detector with graphite monochromated Cu K α X-rays. Diffraction data were collected as 2° oscillation images and reduced to integrated intensities using DENZO⁴². Scaling parameters for each image were calculated with ROTAVATA⁴³ and applied with AGROVATA⁴³. **MIR analysis and refinement.** Selenomethionyl (SeMet) ZAP-NC was crystallized with TML under the same conditions and data collected. Positions of the lead and selenium atoms were determined from the difference Patterson function. Anomalous dispersion measurements were included for both datasets. Heavy-atom parameters were refined, and phases were obtained at 2.8 Å using MLPHARE⁴³. The MIR phases were improved with the program DM⁴³ with a combination of solvent flattening/histogram mapping and phase extension to 2.0 Å. Electron-density maps with MLPHARE and DM phases were calculated, and the polypeptide chain model was built with the program O⁴⁴. SIGMAA⁴³ was used to perform several cycles of phase combination using partial model and experimental phases. Least-squares refinement with simulated annealing was done using X-PLOR⁴⁵. The current model has all residues from Asp 3 to Asn 256 of the protein, all 19 peptide residues, and 113 water molecules, plus one lead and three selenium atoms. TML is bound to Cys 117.

$$^* R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i \times 100.$$

$$^{\dagger} R_c = \sum \|F_{\text{ph}} \pm F_p\| - F_{\text{calc}} / \sum F_{\text{ph}} \pm F_p \text{ for centric reflections.}$$

$$^{\ddagger} \text{Phasing power, } F_p/E, \text{ where } E = \text{r.m.s. lack of closure error.}$$

$$^{\S} R_{\text{value}} = \sum \|F_{\text{obs}}\| - |F_{\text{calc}}| / \sum |F_{\text{obs}}| \times 100.$$

$$^{\parallel} \text{Subset of data (10\%)} \text{ was excluded from refinement and used for free } R\text{-value calculation. All data with } F > 2\sigma \text{ were used for refinement.}$$

loop. Owing to the size of this pocket, ζ Leu 7 is contacted directly by only 5 residues: Tyr 211, Ile 223, Gly 226, Gly 245 and Leu 246. The depth of this pocket is partly due to the presence of a leucine in helix B that is occupied by a tyrosine in many other SH2 domains²¹. A second contribution to the overall shape of the pY + 3 pocket is provided by a repositioning of the β -turn in the EF loop. In complexes of isolated SH2 domains, this loop is involved in forming the steep solvent-exposed wall of the pocket. In ZAP-C, the EF loop slides toward strand D to allow the remainder of the peptide to continue on its path toward ZAP-N.

Binding of Intermotif (-NLGRREE-)

The peptide segment that separates the pYXXL motifs in ζ_1 consists of seven amino acids that make the bulk of their contacts to ZAP-C. Because nearly a full turn of an α -helix begins at ζ Asn 8 and continues to ζ Arg 12, many contacts for this sequence are intrapeptide. ζ Asn 8 and ζ Arg 12 both contact ZAP-C by several direct and water-mediated hydrogen bonds. The side chains of ζ Leu 9 and ζ Arg 12 close off the pY + 3 pocket of ZAP-C.

Two glutamate residues complete this segment of ζ_1 . As the pY-1 and pY-2 residues of the second pYXXL motif, they constitute the first contacts to ZAP-N. ζ Glu 13 is involved in a direct hydrogen bond through its side-chain carboxyl to the side-chain amino group of Lys 242 (ZAP-C α B), which is an integral part of the phosphotyrosine pocket of the N-terminal SH2 domain. ζ Glu 13 also maintains van der Waals contact to Lys 242, as well as to Tyr 238 (ZAP-C α B) and Arg 17 (ZAP-N α A), which also contribute to the N-terminal pY pocket. ζ Glu 14 maintains the characteristic pY-1 contacts.

Binding of motif-2 (-pYDVL-)

The most remarkable and unique feature of the complex between ZAP-NC and ζ_1 is the recognition pocket of ζ pTyr 15, which is composed of residues from both SH2 domains (Fig. 3b, c). The impinging of ZAP-C on ZAP-N sequesters ζ pTyr 15 in a deep tunnel. The side chain of ζ pTyr 15 makes van der Waals contacts to Arg 41 (BC loop), Val 47 (β C), His 58 (β D) and Pro 60 (β D). The side chain of Arg 17 is positioned over the aromatic ring of ζ pTyr 15, forming an amino-aromatic contact in addition to bridging the carbonyl of the pY-1 residue to the phosphate oxygens of ζ pTyr 15. The phosphate group is closely associated with the side chains of Tyr 238 and Lys 242 (both donated by ZAP-C α B), Arg 17 (α A) and Arg 37 (β B), forming a total of six direct hydrogen bonds. Six water-mediated hydrogen bonds exist between the phosphate group and Arg 17 (α A), Cys 39 (β C), Leu 40 (BC loop), Arg 41 (BC loop) and Lys 242 (ZAP-C α B). Mutagenesis experiments are required to determine the relative importance of each residue in this interface. Four water molecules contribute to this extensive network; their presence may be a consequence of the intrusion of ZAP-C onto residues of the BC loop.

The pY+1 and pY+2 residues (ζ Asp 16 and ζ Val 17) make contacts that are characteristic of these positions in other SH2 complexes¹⁹⁻²¹. ζ Leu 18 resides in a hydrophobic pocket that is of similar dimension to the pY+3 pockets observed in high-affinity peptide complexes with Src family SH2 domains. Contact with several hydrophobic residues is evident: β D contributes Phe 59; interaction with the EF loop involves Ile 71, Ala 72, Gly 73 and Gly 74; helix B presents Tyr 87; and the BG loop makes contact by means of Gly 93 and Leu 94.

Unlike the extensive contact area between ζ_1 and ZAP-NC, the total interaction area between ZAP-N and ZAP-C is small, measuring only $\sim 200 \text{ \AA}^2$. The interface that is exclusively between ZAP-N and ZAP-C consists mostly of water-mediated hydrogen bonds, although van der Waals contacts do exist (Fig. 4). Given that the total interface that is exclusive to the two SH2

domains may not exist without the peptide, the inter-SH2 spacer is likely to stabilize the appropriate orientation for tandem binding by allowing only minor displacements. In isoelectric focusing gels, uncomplexed ZAP-NC exists as multiple bands which collapse into a single band when ζ_1 is added (J.L.K. and M.H.H., unpublished observation). This microheterogeneity observed with uncomplexed ZAP-NC may be indicative of conformational variability.

Comparison with other complexes

Despite the low sequence identity (33%) between ZAP-N and ZAP-C, the similarity in overall fold is notable. Side-chain positions are remarkably well conserved between the two domains. The overall backbone root mean square (r.m.s.) deviation is 1.07 $\text{\AA}$; the same measurement for ZAP-N or ZAP-C to Src family SH2 domains is typically 1.50 $\text{\AA}$, although the percentage of sequence identity is similar. As reported for previous structures of SH2 domains^{19,20,23}, the loop regions display the largest positional variance.

Although each pYXXL motif of ζ_1 resides in a similar backbone conformation in the complex, the orientation of the phosphotyrosines varies between ZAP-N and ZAP-C. The aromatic ring of ζ pTyr 4 superimposes remarkably well with the pTyr in both Lck¹⁹ and v-Src²⁰. For ζ pTyr 15, however, the ring is repositioned 0.7 $\text{\AA}$ towards the guanidinium of Arg 17 and slips 0.8 $\text{\AA}$ away from strand D. This is probably due to the direction taken by ζ_1 as it moves into the ZAP-C domain, as well as the strong hydrogen-bonding interactions between the phosphate group and Tyr 238 and Lys 242 on ZAP-C. Both pTyr pockets of ZAP-NC are large enough for several water molecules to be included; this enlargement relative to other SH2 domains is due to the repositioning of the BC loop. The extended position for the BC (phosphotyrosine binding) loop observed for both SH2 domains of ZAP-70 has been observed previously for uncomplexed SH2 domains, and has been described as a hinge in the binding of tyrosine phosphorylated and phosphonated peptides^{20,23}. Although the loop is reorientated, the internal conformation for the BC loop is strongly maintained.

Finally, in comparison with all other crystal structures of complexes of SH2 domains, a significant number of water molecules are present in ZAP-NC. Several are involved in bridging phosphotyrosine to the protein. Additionally, a large number of buried water molecules exist in all interfaces.

Biological significance

The structure of the tandem SH2 domains of ZAP-70 in complex with a component of the ζ -chain of the TCR provides a view at the molecular level into the intracellular machinery of the TCR (Fig. 5). This structure suggests that the SH2 domains do not function independently, and that interactions between the domains are critical in the recognition of the TCR. The structure is key to the interpretation of genetic and biochemical data, and provides a framework for exploring the mechanism of action of ZAP-70.

The tandem SH2 domains of ZAP-70 exhibit high selectivity for the phosphorylated ζ - and ϵ -subunits of the TCR, but isolated SH2 domains from other proteins bind to many tyrosine-phosphorylated proteins in total cell extracts⁵. Although ZAP-NC exhibits high affinity for doubly phosphorylated ITAMs and selectivity for the ζ - and ϵ -chains, the individual SH2 domains of ZAP-70 have not been found to bind appreciably to phosphorylated peptides⁵. In addition, ZAP-NC binds to mono-phosphorylated ITAM-based peptides with affinities that are 100–1,000 times lower than for the corresponding doubly phosphorylated ones^{11,13,27}. The selectivity of ZAP-70 for doubly phosphorylated ITAM sequences appears to be a consequence of multiple structural features. The distance between the two pYXXL motifs of the ζ - or ϵ -chain provides properly spaced partners for a pair of SH2 domains that are tethered in close

association by an inter-SH2 coiled coil. Consequently, high-affinity binding is due to the dramatic entropic advantage that stems from the bidentate interaction. The structural manifestation of this is also the most remarkable feature of the complex between ZAP-NC and ζ_1 , that is, the convergence of residues from both SH2 domains to enmesh pTyr 15.

It has so far been assumed that SH2 domains adopt their native fold when extracted from their natural molecular context

and possess their full ability to recognize and bind to phosphorylated proteins¹⁵. Here we present structural evidence that the N-terminal SH2 domain of ZAP-70, if expressed in isolation, is incomplete. The groove-like nature of the pY pocket of ZAP-C suggests that this domain may also require contributions from neighbouring domains or proteins.

Studies involving mutational alterations in ITAM sequences of TCR subunits show that one additional residue between

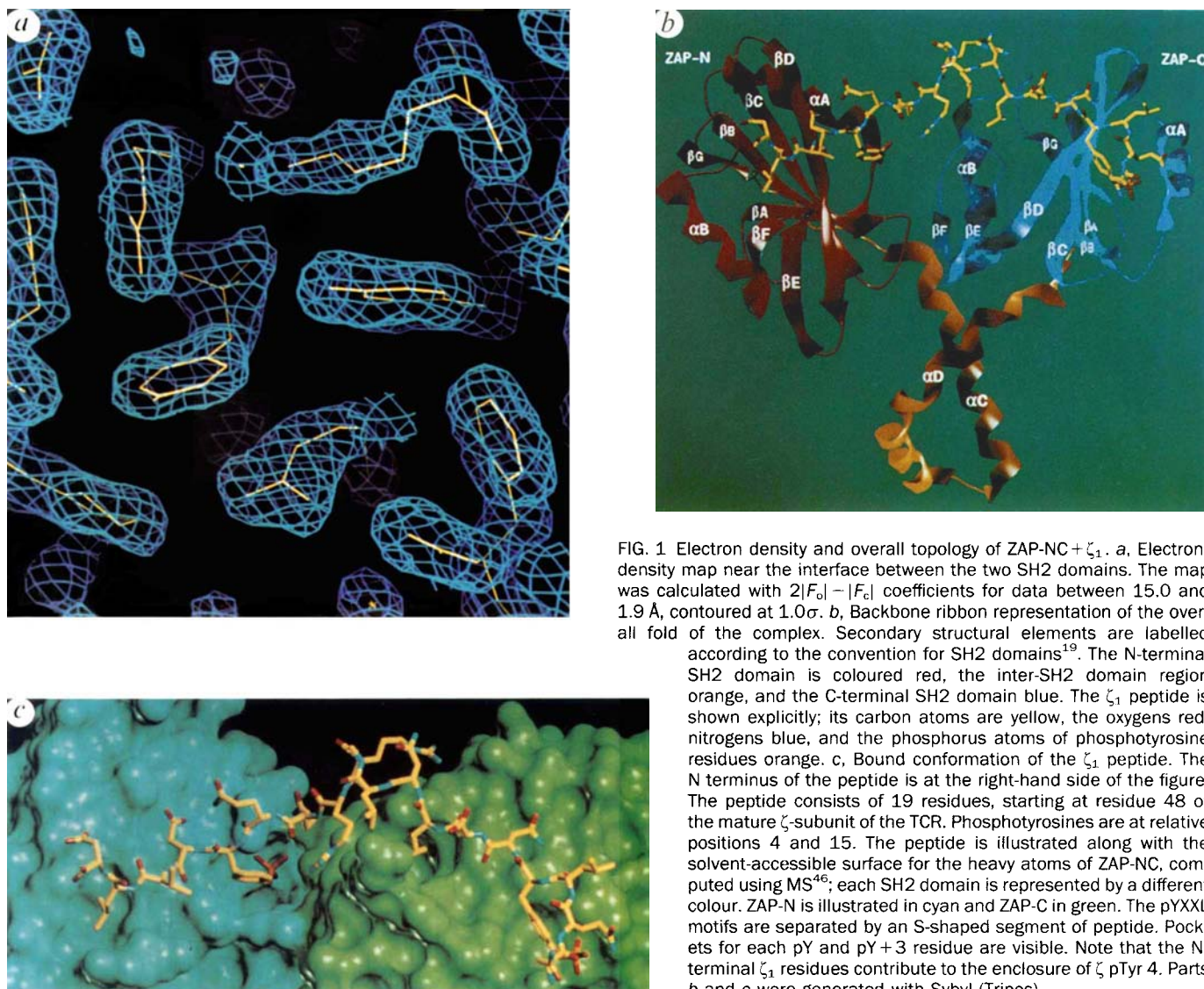


FIG. 1 Electron density and overall topology of ZAP-NC + ζ_1 . **a**, Electron-density map near the interface between the two SH2 domains. The map was calculated with $2|F_o| - |F_c|$ coefficients for data between 15.0 and 1.9 Å, contoured at 1.0σ . **b**, Backbone ribbon representation of the overall fold of the complex. Secondary structural elements are labelled according to the convention for SH2 domains¹⁹. The N-terminal SH2 domain is coloured red, the inter-SH2 domain region orange, and the C-terminal SH2 domain blue. The ζ_1 peptide is shown explicitly; its carbon atoms are yellow, the oxygens red, nitrogens blue, and the phosphorus atoms of phosphotyrosine residues orange. **c**, Bound conformation of the ζ_1 peptide. The N terminus of the peptide is at the right-hand side of the figure. The peptide consists of 19 residues, starting at residue 48 of the mature ζ -subunit of the TCR. Phosphotyrosines are at relative positions 4 and 15. The peptide is illustrated along with the solvent-accessible surface for the heavy atoms of ZAP-NC, computed using MS⁴⁶; each SH2 domain is represented by a different colour. ZAP-N is illustrated in cyan and ZAP-C in green. The pYXXL motifs are separated by an S-shaped segment of peptide. Pockets for each pY and pY + 3 residue are visible. Note that the N-terminal ζ_1 residues contribute to the enclosure of ζ pTyr 4. Parts **b** and **c** were generated with Sybyl (Tripos).

FIG. 2 Sequence alignments for selected SH2 domains³⁶. Boxed areas indicate segments used for measuring the 'full' backbone r.m.s. deviation reported in the text; unboxed areas are excluded from the calculation owing to the presence of gaps for one or more of the sequences. Notation below the alignments indicate structurally conserved regions, and use the nomenclature reported previously¹⁹. Shaded regions indicate the secondary structural elements in ZAP-NC. Src, avian Src; Lck, human p56^{lck}; Syp-N, N-terminal SH2 of murine Syp phosphatase (PTP1D); ZAP-N and ZAP-C, N- or C-terminal SH2 of human ZAP-70.

Src:	WYF	GKI	TRRESERLLL	NPENPRG	TFLVRES	ETTKG	AYCLSVSD	FDNAKGL
Lck:	WFF	KNL	SRKDAERQLL	APGNTHG	SFLIRES	ESTAG	SFSLSVRD	FDQNQGE
Syp-N:	WFH	PNI	TGVEAENLLL	TRG	VDG	SFLARPS	KSNPG	DFTLSVRR
ZAP-N:	FFY	GSF	SRAEAEHLK	LAGMADG	LFLLRQC	LRLSLG	GYVLSLVH	D V
ZAP-C:	WYH	SSL	TREEAERKLY	SGAQTG	KFLLRPR	KE	QGYALSIIY	GK
	β A		α A		β B		β C	

Src:	NVKHYKI	RKL	DSG	GFYI	TSR	TQF	N	SLQQVLVAYYSKH	ADGL	CHRLT	TVC
Lck:	VVKHYKI	RNL	DNG	GFYI	SPR	ITF	P	GLHELVRHYTNA	SDGL	CTRLS	RPC
Syp-N:	AVTHIKI	QNT	GD	YYDL	YGG	EKF	A	TLAELVQYYMEH	HGQLKEKNGD	VIELK	YPL
ZAP-N:	RFHHFPI	ERQ	LNG	TYAI	AGG	KAH	C	GPAELCEFYSRD	PDGL	PCNLR	KPC
ZAP-C:	TVYHYLI	SQD	KAG	KYCI	PEG	TKF	D	TLWQLVEYLKLLK	ADGL	IYCLK	EAC
	β D		β D'	β E		β F		α B		β G	

pYXXL motifs is tolerated, but a deletion of two amino acids drastically reduces ZAP-70 binding and eliminates production of interleukin (IL)-2¹¹. Therefore the distance between the two pYXXL motifs is important for association and signalling. The relative contribution of each residue of the ITAM was explored by alanine scanning²⁸. Only the replacement of each pY or pY + 3 residue eliminates signalling completely, as measured by IL-2 production. Apart from these residues, the specific sequence of the ITAM is less important for binding than the distance between pYXXL motifs.

The inter-SH2 region constrains the SH2 domains of ZAP-70 within a distance that allows their association. However, because a significant portion of the antiparallel helices is directed away from the SH2 domains, we speculate that this region may also be important for inter- or intramolecular interactions that regu-

late the kinase activity of ZAP, dimerization during TCR clustering, or association with other cellular proteins. Coiled coils are commonly involved in protein-protein interactions²⁹. The inter-SH2 domain may inhibit the catalytic activity of ZAP-70 through intramolecular interactions that are relieved upon binding of the SH2 domains to the ITAM. Experiments with phosphatidylinositol-3-OH kinase (PI(3)K)³⁰ and Syk³¹ support this model. Activation of PI(3)K is induced *in vitro* by a phosphotyrosine peptide from the platelet-derived growth factor (PDGF)- β receptor³⁰. Similarly, phosphorylated ITAM peptides derived from the γ -subunit of the IgE receptor increase Syk kinase activity by five- to tenfold^{32,33}. There is also precedent for involvement of such inter-SH2 regions in intermolecular associations. The inter-SH2 region of the p85 subunit of PI(3)K, which is predicted to form a coiled coil, is necessary and sufficient for

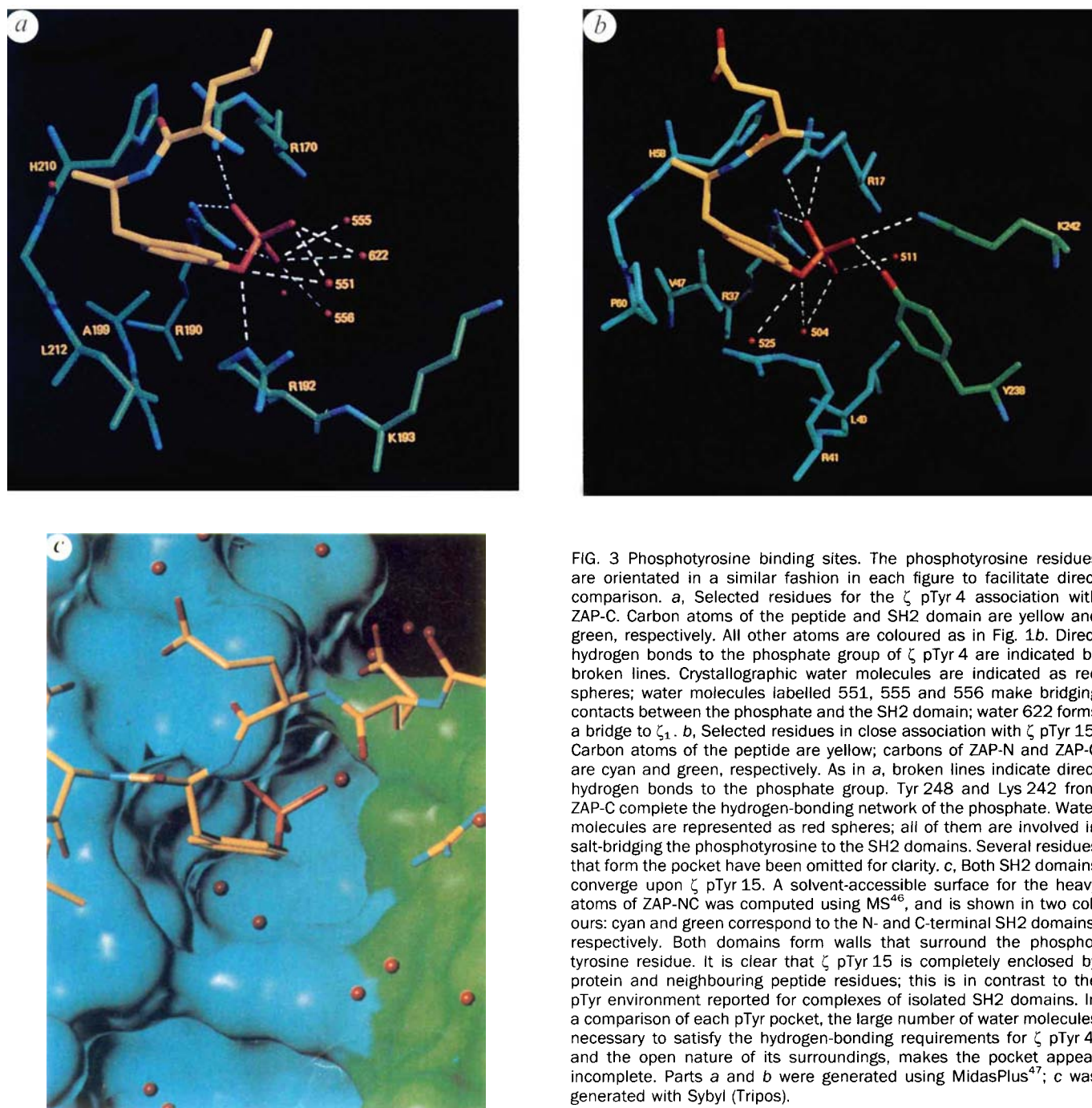
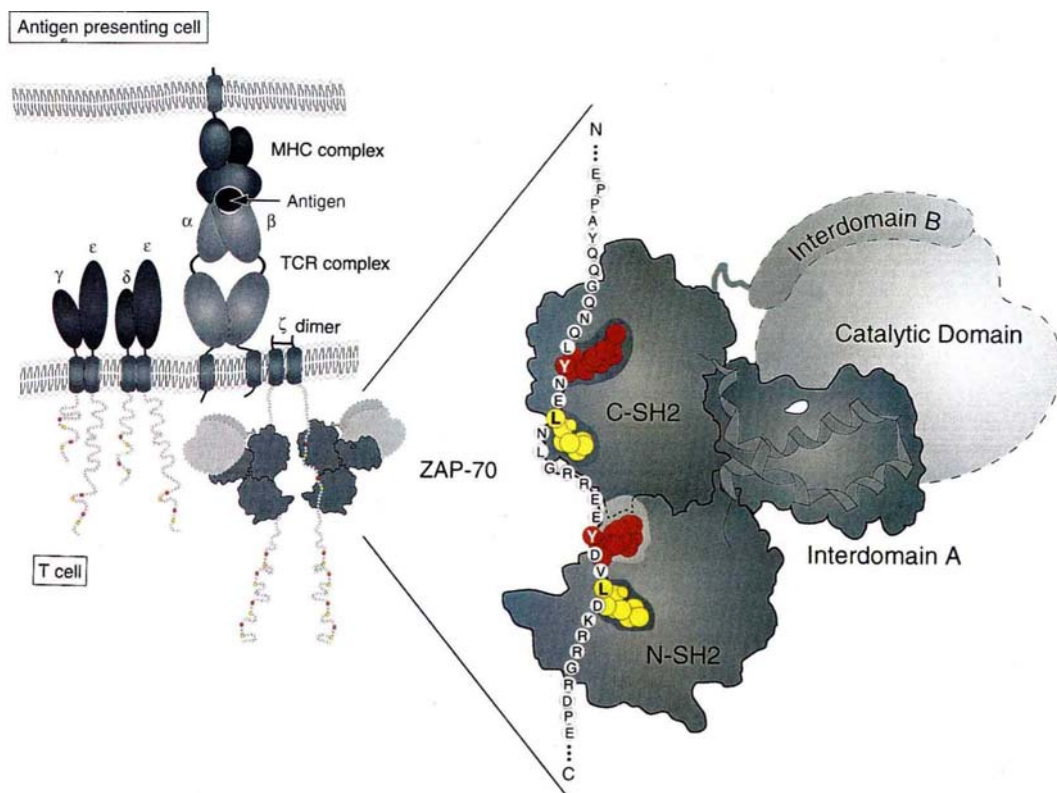


FIG. 3 Phosphotyrosine binding sites. The phosphotyrosine residues are orientated in a similar fashion in each figure to facilitate direct comparison. *a*, Selected residues for the ζ pTyr 4 association with ZAP-C. Carbon atoms of the peptide and SH2 domain are yellow and green, respectively. All other atoms are coloured as in Fig. 1*b*. Direct hydrogen bonds to the phosphate group of ζ pTyr 4 are indicated by broken lines. Crystallographic water molecules are indicated as red spheres; water molecules labelled 551, 555 and 556 make bridging contacts between the phosphate and the SH2 domain; water 622 forms a bridge to ζ_1 . *b*, Selected residues in close association with ζ pTyr 15. Carbon atoms of the peptide are yellow; carbons of ZAP-N and ZAP-C are cyan and green, respectively. As in *a*, broken lines indicate direct hydrogen bonds to the phosphate group. Tyr 248 and Lys 242 from ZAP-C complete the hydrogen-bonding network of the phosphate. Water molecules are represented as red spheres; all of them are involved in salt-bridging the phosphotyrosine to the SH2 domains. Several residues that form the pocket have been omitted for clarity. *c*, Both SH2 domains converge upon ζ pTyr 15. A solvent-accessible surface for the heavy atoms of ZAP-NC was computed using MS⁴⁶, and is shown in two colours: cyan and green correspond to the N- and C-terminal SH2 domains, respectively. Both domains form walls that surround the phosphotyrosine residue. It is clear that ζ pTyr 15 is completely enclosed by protein and neighbouring peptide residues; this is in contrast to the pTyr environment reported for complexes of isolated SH2 domains. In a comparison of each pTyr pocket, the large number of water molecules necessary to satisfy the hydrogen-bonding requirements for ζ pTyr 4, and the open nature of its surroundings, makes the pocket appear incomplete. Parts *a* and *b* were generated using MidasPlus⁴⁷; *c* was generated with Sybyl (Tripos).

FIG. 5 Schematic view of ZAP-70 bound to ζ_1 of the activated TCR. Left, activation of T cells is initiated by association of the TCR with a peptide antigen bound to the major histocompatibility complex (MHC) on an antigen-presenting cell. TCR-MHC association stimulates phosphorylation of TCR subunits on tyrosines (shown in red) within the ITAMs. ZAP-70 binds to the phosphorylated ITAM by means of its SH2 domains (residues 1–259). The positions of the other domains of ZAP-70, referred to as interdomain B (residues 260–310) and catalytic domain (residues 311–620), have not been determined, and their positions as shown are hypothetical. Two ZAP-70 molecules could bind to the activated TCR complex, as the ζ -subunit is present as a disulphide-linked dimer. Right, schematic representation of the complex between the SH2 domains of ZAP-70 in complex with the doubly phosphorylated ζ_1 ITAM. The SH2 domains of ZAP-70 make extensive contacts with ζ_1 . The primary determinants of binding are the phosphotyrosine (red) and leucine (yellow) residues of two pYXXL sequences within an ITAM. The structure reveals a unique binding pocket for the pY of the C-proximal pYXXL motif in the interface between the two SH2



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interaction of p85 with the p110 catalytic subunit of PI(3)K³⁴. The inter-SH2 region could be involved in interactions with proteins that regulate ZAP-70, such as Lck or Fyn, or that are substrates for ZAP-70. As there is at least one tyrosine in the inter-SH2 region that is phosphorylated by Lck *in vitro*³⁵, such interactions could involve SH2 domains from other proteins.

We anticipate that Syk will also exhibit these structural features as a result of the functional similarities and its sequence identity of 57%. Syk is expressed in several types of haematopoietic cells, and functions in mast cells and B cells by binding to ITAM sequences in the cytoplasmic domains of IgE and B-cell receptors, respectively³¹. Most of the residues in ZAP-NC that contact pTyr 15 are conserved in the corresponding positions in Syk³⁶. The N-terminal SH2 domain of Syk does not bind to phosphotyrosine ligands or to phosphotyrosine affinity columns³⁷, which suggests that this phosphotyrosine site also requires the C-terminal SH2 domain for it to form a complete pocket. It is known that doubly phosphorylated peptides derived from the γ ITAM of the IgE receptor induce Syk activation³². We therefore infer that this complex of ZAP-NC with ζ_1 represents the conformation of the SH2 domains in the activated kinase.

ZAP-70 is an attractive target for the development of potent immunosuppressive drugs. It is required for T-cell-mediated immune responses in humans, and its loss does not affect other tissues⁸. Thus ZAP-70 antagonists would inhibit T-cell function specifically and avoid the well-documented toxicity of currently used immunosuppressives. Tacrolimus (FK506) and cyclosporin^{38, 41} exhibit side effects that limit their application largely to organ transplant rejection³⁸. Therefore new immunosuppressive drugs with lower toxicity are needed to expand the routine use of such therapies to autoimmune diseases.

One approach to the inhibition of T-cell activation is to develop non-peptidic molecules that prevent ZAP-70 association with the TCR. Because both ZAP-70 SH2 domains are required for TCR coupling, a membrane-permeable inhibitor that binds with high affinity to either SH2 domain of ZAP-70 or that alters the structure of the interface should inhibit ZAP-70 function in activated T cells. The crystal structure provides the molecular details of ZAP-70 interactions with the TCR. The unique structural features of each SH2 domain and the unanticipated interfaces can be exploited for structure-based design of specific ZAP-70 ligands and structurally biased combinatorial libraries. □

Received 1 June; accepted 13 July 1995.

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ACKNOWLEDGEMENTS. We thank S. L. Schreiber, J. S. Brugge and M. Weigle for comments and discussions. Coordinates will be deposited in the Brookhaven Protein Data Bank.

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Deficit of distant X-ray-emitting galaxy clusters and implications for cluster evolution

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CLUSTERS of galaxies are the largest gravitationally bound systems in the Universe and therefore provide important constraints on the formation and evolution of large-scale structure. Cluster evolution can be inferred from observations of the X-ray emission of the gas in distant clusters, but interpreting these data is not straightforward. In a simplified view, clusters grow from perturbations in the matter distribution, and the intracluster gas is compressed and shock-heated by the gravitational collapse¹. If the gas is in hydrostatic equilibrium the resulting X-ray emission is related in a simple way to the evolving gravitational potential. But if processes such as radiative cooling or pre-collapse heating of the gas are also important, the X-ray evolution will be strongly influenced by the thermal history of the gas. Here we present the results of a faint flux-limited sample of X-ray selected clusters observed by Rosat. Very few distant clusters have been identified, and their redshift distribution seems to be inconsistent with simple models based on the evolution of the gravitational potential. Our results thus suggest that radiative cooling or non-gravitational heating of intracluster gas must be important in the evolution of clusters.

Clusters of galaxies are visible as concentrations of physically associated galaxies and as regions of extended X-ray emission. But optical surveys for distant clusters are hampered by the likely superposition of physically separate systems along the line of sight. This makes it difficult to use such studies to quantify cluster evolution reliably. In contrast, X-ray studies are unaffected by such difficulties and thus offer a direct measure of the evolution. But the flux-limited surveys so far have only been able to detect the most luminous examples at cosmologically significant distances. Two groups^{2,3} have reported a decrease in the amplitude of the X-ray luminosity function (XLF) with redshift. Edge *et al.*², studying a combined sample from several surveys, found a deficit in the volume density of luminous clusters in the range $z \sim 0.1$ – 0.2 when compared with predictions based on the local XLF. The Einstein Extended Medium Sensitive Survey (EMSS)^{3,4} showed a similar trend at a fainter limit. If we accept that the power spectrum of cosmological density fluctuations is similar to that of the Cold Dark Matter model⁵, these results are in clear contradiction of the simplest models of evolution, where the intracluster gas evolves with the cluster potential in a self-similar fashion. In such models the typical cluster masses decrease with redshift as their densities increase. The X-ray luminosity is more strongly related to the density (the balance being set by the choice of power spectrum) and therefore

TABLE 1 Relative likelihood values ($0.2 < z < 0.7$)

Model	RIXOS likelihood	EMSS likelihood
Self-similar ($n \approx -2$)	19.3	0.0
No evolution	8.7	9.2
Fixed $L_x - T_x$	0.0 ($n = -1.5$)	0.4 ($n = -0.8$)

the net prediction is a higher number of clusters of a given luminosity in the past or, conversely, an increase in the amplitude of the XLF with redshift. The EMSS data for the highest redshift bin sampled ($\bar{z} = 0.33$) show a steeper slope in the XLF. If this trend were maintained to lower luminosities, surveys of yet fainter objects would reveal a large increase in the number of subluminoous clusters at intermediate redshifts over expectations based on the local XLF. The fainter flux limit achievable with Rosat⁶ can thus test the form of cluster X-ray evolution as well as extend previous tests to higher redshift.

The sample presented here was derived from the Rosat International X-ray Optical Survey (RIXOS), a project aimed at producing a catalogue of optical identifications for ~ 400 X-ray sources found in 81 northern Rosat fields observed with the position-sensitive proportional counter (PSPC). Fields at high Galactic latitude ($b > +28^\circ$) were selected with exposure times > 8 ks achieving a limiting flux optimized for wide-area optical follow-up. To avoid degradation of the point-spread function at large radii, and interference from the PSPC window support structure, only sources within $17'$ of the field centre were chosen. In total, 385 X-ray sources were catalogued in 81 fields over 20.4 deg^2 to a limiting flux of $f_x \geq 3.0 \times 10^{-14} \text{ erg s}^{-1} \text{ cm}^{-2}$ in the 0.5–2.0 keV energy band (Carrera *et al.*, manuscript in preparation).

Optical imaging and spectroscopic follow-up were carried out at La Palma with time allocated under the international programme supported by the Comité Científico Internacional. The results presented here refer to a subset of 59 randomly selected fields for which complete identifications were secured for 264 sources. In considering completeness for the analysis below, allowance has been made for a few bright (2–14 mag) stars very close to the X-ray position whose low-resolution spectra ($9 \text{ \AA}$) are insufficient to reveal evidence of chromospheric activity. A further 14 sources (5%) have no convincing spectroscopic identification; however, in all but three cases, there is only a single isolated candidate (presumably a BL Lac) within the X-ray error box. For the remaining 3 sources, only one could conceivably be a cluster; a deep image in good conditions reveals some very faint sources unfortunately beyond reach of 4-m spectrographs.

Figure 1a shows the redshift distribution of the 13 identified RIXOS clusters with redshift > 0.2 . As discussed above, there is at most one distant candidate, which we tentatively place at $z = 1$ on the basis of the apparent R magnitudes of the brightest galaxies. Regardless of this uncertainty in the high- z tail, we demonstrate that pushing to a fainter flux limit has revealed neither an abundant population of distant luminous clusters nor numerous subluminoous clusters at intermediate redshift.

Figure 1a also shows predicted redshift distributions for the RIXOS selection criteria calculated by integrating an appropriate XLF over luminosity and redshift in three physically interesting cases. Each prediction incorporates a maximum-likelihood fitting of the cluster emission to yield the detected flux. The model profile is determined by convolving a standard $\beta = \frac{2}{3}$ profile⁷ (with a core radius of $r_0 = 250 \text{ kpc}$) with a gaussian approximation of the PSPC point-spread function. Calculations are corrected to the 0.5–2.0 keV band, assuming $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0.5$. Table 1 compares, for the three cases, the relative likelihoods of accounting for the data obtained in this survey. A unique feature of the RIXOS survey, however, arising from its faint flux limit, is its ability to distinguish between different evolutionary models that are equally consistent with the earlier EMSS data observed to a brighter flux limit. To

TABLE 2 RIXOS and EMSS flux limits and area coverages

Survey	Flux limit (10^{-14} erg cm $^{-2}$ s $^{-1}$)	Area coverage (deg 2)
RIXOS	3.0	14.9
EMSS	3.6	0.09
EMSS	6.2	6.4
EMSS	7.4	15.1

Comparison of the RIXOS and EMSS surveys. The entire area covered by RIXOS was surveyed to the same limiting flux in the Rosat 0.5–2.0-keV band. But the area covered by the EMSS survey was searched at different depths in the Einstein 0.3–3.5-keV band. For this comparison the EMSS flux limits¹³ have been converted to the Rosat band. The relative areas surveyed depend on the cluster temperature and redshift assumed, and are also affected by different detection procedures, which are similarly redshift-dependent. This table has been calculated for a cluster of temperature $T_X = 3$ keV at a redshift of $z = 0.3$. For the same area coverage, RIXOS is a factor 2.5 deeper than the EMSS in this case.

illustrate this point, we also compare our evolutionary predictions with published data for the EMSS survey^{4,8} in Fig. 1b. Table 2 compares the area covered and the flux limits achieved in both surveys.

Our first prediction is that expected for a non-evolving cluster population based upon the local XLF of Edge *et al.*². Although this model is inconsistent with the earlier X-ray results^{2,3}, it is still an important null hypothesis given that optical surveys⁹ have failed to detect any strong evolution in the volume density of rich clusters to $z \sim 0.5$. However, in this case, given our faint limiting flux, we ought to detect X-ray clusters readily to very high redshifts. Even including the tentative candidate at $z \sim 1$, the Poisson probability of finding a single cluster beyond $z = 0.6$, when 7.9 are expected in the no-evolution case, is 0.3%. Furthermore, the model is in qualitative disagreement with the steep decline in the number of clusters observed within $0.2 < z < 0.7$. The RIXOS survey therefore confirms the marked decline in the volume density of luminous clusters with redshift seen in the earlier surveys.

The next model we consider assumes that the slope of the initial spectrum of density fluctuation is shallow ($n \approx -2$). In this case, the initial density perturbations that lead to the formation of clusters are small and the gravitational collapse of the most massive clusters has occurred only recently. At high luminosities, this model produces a decrease in the number of high-redshift clusters, but this is counterbalanced by a rapid increase (with redshift) in the number of subluminoous clusters. To generate the model prediction we apply the self-similar scaling laws described by Kaiser¹⁰. These laws encapsulate the physical principles governing the evolution of cluster gravitational potentials. Thus, unless the intracluster gas responds to additional physical constraints, we can use self-similarity to recalculate the high-redshift XLF accurately by rescaling and renormalizing the present-day equivalent of Edge *et al.*². This self-similar model does not naturally reproduce the observed X-ray luminosity–temperature relation¹¹; however, the model (with the flat spectral index) can account for the evolutionary trends seen in the EMSS survey. Significantly, the fainter RIXOS survey does not detect as many intermediate-redshift clusters as expected in this model. Specifically, 32.9 clusters would be expected within $0.2 < z < 0.7$ compared with only 13 observed, a probability of 0.01%.

Our final model examines the situation if the self-similarity of Kaiser's scaling laws XLF is broken. Physical processes such as gas cooling, gas stripping, feedback from galaxy formation or winds could each break this scaling. In an open Universe, it is possible that the changing rate at which gas falls into the cluster potential could produce a similar effect. Within the variety of possible evolutionary models that might be proposed, we choose one that is intended to reproduce qualitatively the 'preheated'

intracluster medium model proposed by Kaiser¹⁰. In such a model the intracluster gas is heated at some early epoch acquiring a high characteristic entropy. This might arise, for example, via multiple supernova explosions, occurring at the epoch of galaxy formation, which expel energy into the primordial intracluster medium. Thereafter the central gas, which produces most of the emission, remains adiabatic through the collapse process. The resulting X-ray luminosity–temperature relation has a slope consistent with its present-day value¹¹, which does not depend on redshift. We consider the spectral index of the density fluctuations as a free parameter that is adjusted to give the best fit to the data. With a spectral index of $n = -1.5$, a good fit to the RIXOS results can be obtained (Table 1). A likelihood ratio test¹² based on the RIXOS data with $0.2 < z < 0.7$ shows an almost 20-fold improvement for the 'preheated' intracluster model, in which the L_X – T_X relation remains fixed in redshift, over the 'self-similar' case (formally corresponding to >99.99% confidence). Likewise, the ratio compared with the no-evolution

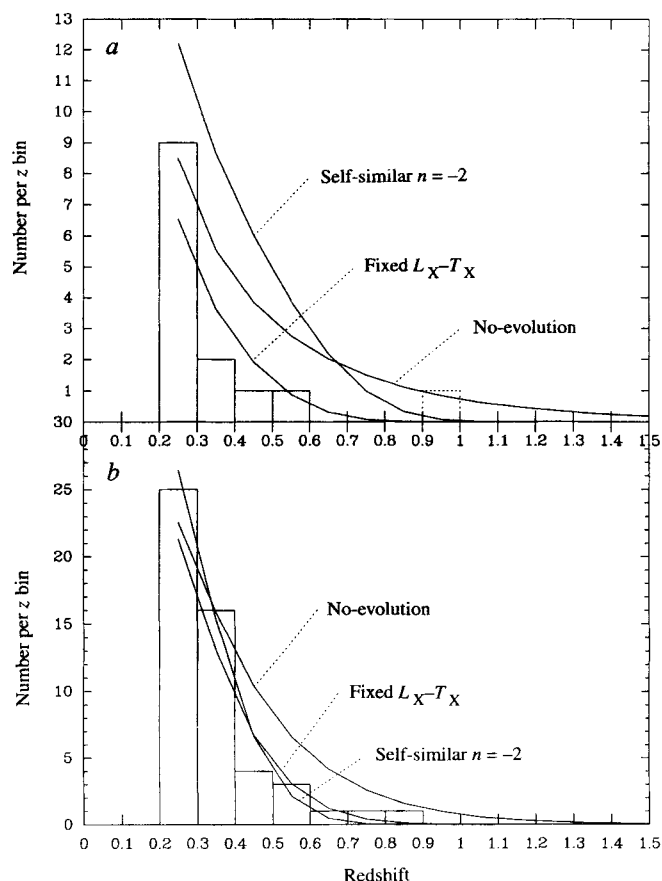


FIG. 1 Redshift distribution for complete samples of faint X-ray selected clusters from the RIXOS survey (a) and the EMSS^{4,8} survey (b). The dotted datum in the RIXOS survey represents a candidate cluster with an assigned redshift of $z = 1.0$ (see text). Smooth curves show the predicted redshift distributions for several evolutionary models: (i) a non-evolving cluster population (based on the local XLF of Edge *et al.*²); (ii) a self-similar model with $n = -1.95$ spectral index; and (iii) the best-fitting preheated intracluster medium model (with spectral index parameter $n = -1.5$ and $n = -0.8$ in a and b respectively). Although both surveys have detected nearby clusters, only clusters with redshift $z > 0.2$ are shown. For clusters at lower redshifts, uncertainties in modelling the cluster profile and the XLF at very low luminosities ($L_X \leq 5 \times 10^{42}$ erg s $^{-1}$) make comparison with model predictions unreliable. Despite the smaller sample of the RIXOS survey (because of its smaller total area coverage), RIXOS is able to detect lower luminosity clusters (see Table 2). In this way, it probes the evolution of the faint end of the XLF, enabling discrimination between evolutionary models that were both compatible with the earlier EMSS survey.

case is 8.7 (99.7% confidence). We note a further important point in this comparison: a different spectral parameter is required to fit the RIXOS and EMSS data sets (see Table 1). This most probably reflects the greater fragility of low-luminosity clusters to the effects of preheating—a subtlety that has not been incorporated into our simple model.

Notwithstanding the small sample obtained in the RIXOS survey compared with the extensive EMSS survey, by probing to fainter flux limits our data demonstrate two important results. First, there remains a substantial deficit of high-redshift clusters compared with no-evolution expectations, confirming and strengthening conclusions based on earlier studies. Second, at intermediate redshift we have penetrated sufficiently far down the XLF to distinguish between two evolutionary models that were both consistent with the previous data. We do not observe the expected increase in the number density of subluminal clusters predicted in the case where the XLF evolves according to a simple self-similar scaling law. It appears that an additional hydrodynamical ingredient is required to break this scaling. By defining the form of the evolution of the X-ray luminosity function, our data open the way for a theoretical picture that properly accounts for the thermal history of the intracluster gas.

One appealing possibility is that the intracluster gas was heated at an early epoch (perhaps as the result of the galaxy formation process) and has only recently become sufficiently cool to be trapped by the cluster gravitational potential. □

Received 28 December 1994; accepted 4 August 1995.

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ACKNOWLEDGEMENTS. The RIXOS project has been made possible by the award of International Time on the La Palma telescopes by the Comité Científico Internacional. We thank numerous individuals who have contributed and acknowledge discussions with C. Frenk, F. J. Castander was supported by the Instituto de Astrofísica de Canarias and thanks the LAEFF.

Absence of superconductivity in the doped antiferromagnetic spin-ladder compound (La,Sr)CuO_{2.5}

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A SPIN- $\frac{1}{2}$ Heisenberg antiferromagnetic ladder is a model system comprising parallel chains of interacting elemental spins. Spin ladders having an even number of chains have been predicted^{1–6} to exhibit interesting dynamics, including superconductivity, when unoccupied spin sites are introduced along the chain (that is, when the chains are doped with holes). Spin-ladder models thus provide a novel potential mechanism for high-temperature superconductivity in real materials. But unfortunately materials with spin-ladder structure are quite rare^{4–10}, and the few known compounds have not previously been doped successfully with holes. Here we report the high-pressure synthesis of a new hole-doped two-chain ladder compound, La_{1-x}Sr_xCuO_{2.5}. We have observed a marked insulator-to-metal transition in this compound with increased doping, but no superconducting transition down to 5 K. The absence of superconductivity in this material must be reconciled with theoretical predictions based on ideal hole-doped spin-ladder models.

Spin- $\frac{1}{2}$ Heisenberg antiferromagnetic ladders are a unique series of low-dimensional systems in the sense that their electronic properties strongly depend upon whether the number of chains in each ladder is even or odd^{2–4}. Theory predicts for even-chain ladders the presence of an energy gap in the spin excitation spectrum, or a spin gap, and hole-pair formation upon doping that could lead to superconductivity with a novel, purely electronic, mechanism^{1–6}. The physical picture based on a theoretical model called the *t*-*J* model¹¹ is quite straightforward for two-chain ladders: the ground state before doping consists of a set of spin singlets on each rung of the ladder, and excitation can occur only at the expense of a definite energy to create a triplet on one of the rungs. When lightly doped with holes, superconducting pairing correlations can be expected, because it is energetically more favourable to create hole pairs than to create free spins on different rungs. The presence of a spin gap has already

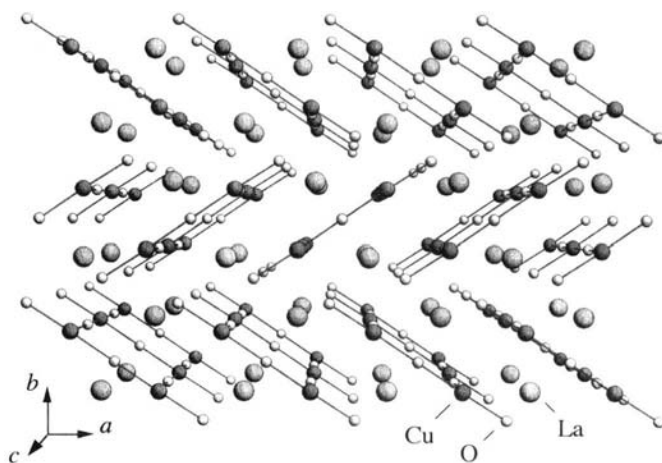


FIG. 1 Perspective view of the crystal structure of LaCuO_{2.5} prepared at high pressure, showing the geometrical arrangement of the Cu O ladders. The coordinate system on the lower left specifies the crystallographic directions.

been confirmed experimentally in a V⁴⁺ compound, (VO)₂P₂O₇ (ref. 12), and a Cu²⁺ compound, SrCu₂O₃ (ref. 13), whereas the possibility of hole pairing, which is apparently more important, has remained uninvestigated because of the difficulty of carrier doping. The present compound La_{1-x}Sr_xCuO_{2.5} would be the first carrier-doped two-chain ladder system.

A base material, La₂Cu₂O₅ (LaCuO_{2.5}), was prepared by the method reported by Cava *et al.*¹⁴. Another series of base materials (La_{1-y}Sr_y)₈Cu₈O_{20-δ} (ref. 15) with *y* = 0.20, 0.25 and 0.30 were prepared from La₂O₃ (dried at 1,000 °C in air), SrCO₃ and CuO by firing at 980 °C in air. Powders of these base materials themselves or appropriate mixtures of them were sealed in gold capsules and treated at 900 °C and 6 GPa for 30 min using a cubic-anvil-type high-pressure apparatus.

The products were characterized by means of powder X-ray diffraction (XRD) and electron diffraction (ED). The XRD patterns changed completely after high-pressure treatment for *x* ≤ 0.20, implying that a structural transformation occurred at high pressure, whereas for *x* = 0.25 and 0.30 the ambient-pressure structure remained intact. Both the XRD and ED patterns indicated the CaMnO_{2.5} structure for 0 ≤ *x* ≤ 0.20, which is one of the prototypes of anion-deficient perovskites¹⁶; ortho-

rhombic, $a \approx \sqrt{2}a_p$, $b \approx \sqrt{2}a_p$, $c \approx a_p$ (a_p is the lattice parameter of the primitive perovskite structure), space group $Pbam$. No impurities were detected. The high-pressure form is denser by about 3% than the ambient-pressure form of $\text{LaCuO}_{2.5}$. In a previous study⁹ $\text{LaCuO}_{2.5}$ with the $\text{CaMnO}_{2.5}$ structure was obtained at ambient pressure by low-temperature reduction of LaCuO_3 prepared at high oxygen pressure. In addition, it was reported that a similar compound appeared below 550 °C for a narrow composition range near $x=0.14$ in $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$ (ref. 10). In the present work, samples of higher quality covering a wide range of Sr content were obtained.

The dense structure of $\text{LaCuO}_{2.5}$ determined by a Rietveld analysis of the powder XRD pattern¹⁷ is illustrated in Fig. 1, and the refined structural parameters are listed in Table 1 for $\text{LaCuO}_{2.5}$ and $\text{La}_{0.8}\text{Sr}_{0.2}\text{CuO}_{2.5}$. Remarkably, the Cu–O sublattice assumes the character of a two-chain ladder along the c axis in Fig. 1, where only Cu–O bonds shorter than 2 Å are shown with solid lines. In the ideal $\text{CaMnO}_{2.5}$ structure, transition metals are coordinated by five oxygen atoms, whereas in the present system the apex oxygens are kept at a distance by a strong Jahn–Teller effect: the Cu–O distances within the ladder are short, ~ 1.95 Å, whereas the Cu–O_{apex} distance is long, 2.29 Å. This unbalanced Cu–O configuration leads us to consider that unpaired electrons are confined to the intra-ladder Cu $d_{x^2-y^2}$ –O $2p_\sigma$ orbitals and that, therefore, strong antiferromagnetic interactions of order 10^3 K would occur only within the ladders. Inter-ladder interactions are further weakened as a result of the approximate orthogonality ($\sim 68.5^\circ$) between adjacent ladders, because electron transfer between mutually orthogonal orbitals is prohibited by symmetry. We may thus assume that each ladder is nearly isolated from the others and provides a quasi-one-dimensional electronic system.

The systematic replacement of La by Sr can be seen in the continuous variation in the lattice parameters shown in Fig. 2. The decrease in a and c indicates a shrinkage of hole-doped ladders: the Cu–Cu distance along the chain (rung) changes from 3.880 Å (3.930 Å) for $x=0$ to 3.865 Å (3.890 Å) for $x=0.20$. In contrast, the increase in b results from the rotation of ladders, which makes the mutual angle larger by about 2° .

A pronounced insulator-to-metal transition has been observed with hole doping in the resistivity (ρ) measurements as shown in Fig. 3a. Non-doped $\text{LaCuO}_{2.5}$ is almost insulating, whereas hole doping systematically decreases resistivity by several orders of magnitude, and finally metallic behaviour down to 5 K occurs for $x=0.20$. The magnitude of resistivity of metallic

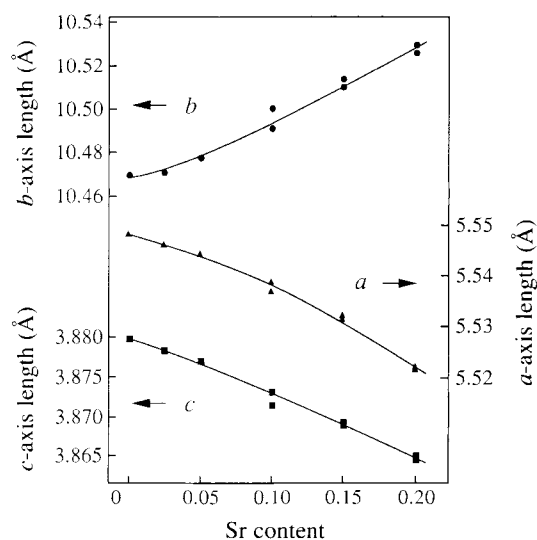


FIG. 2 Variation of the lattice parameters as a function of Sr content in $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$.

TABLE 1 Refined structural parameters for $\text{LaCuO}_{2.5}$ and $\text{La}_{0.8}\text{Sr}_{0.2}\text{CuO}_{2.5}$ *

Atom	Site	x	y	z	B_{iso} (Å ²)
(a) $\text{LaCuO}_{2.5}$ *					
La	4h	0.3122 (1)	0.3602 (1)	0.5	0.28 (2)
Cu	4h	0.2928 (3)	0.1057 (2)	0	0.38 (4)
O(1)	4g	0.2824 (10)	0.1071 (7)	0.5	1.0
O(2)	4g	0.0882 (12)	0.2951 (6)	0	1.0
O(3)	2a	0	0	0	1.0
(b) $\text{La}_{0.8}\text{Sr}_{0.2}\text{CuO}_{2.5}$ †					
La/Sr‡	4h	0.3081 (3)	0.3605 (2)	0.5	0.31 (4)
Cu	4h	0.2874 (7)	0.1069 (4)	0	0.40 (8)
O(1)	4g	0.2960 (21)	0.0959 (13)	0.5	1.0
O(2)	4g	0.0596 (26)	0.2841 (12)	0	1.0
O(3)	2a	0	0	0	1.0

Estimated standard deviations in parentheses refer to the last digit. Isotropic thermal parameters, B_{iso} , were refined for sites La, La/Sr and Cu and fixed for the oxygen sites.

* Space group $Pbam$ (no. 55); $a=5.5482$ (1) Å, $b=10.4677$ (1) Å, $c=3.8801$ (1) Å; $Z=4$ formula unit per unit cell; $R_{\text{wp}}=3.4\%$, $S=0.87$.

† Space group $Pbam$ (no. 55); $a=5.221$ (1) Å, $b=10.5295$ (2) Å, $c=3.8650$ (1) Å; $Z=4$ formula unit per unit cell; $R_{\text{wp}}=3.9\%$, $S=1.10$.

‡ Refined occupancy of Sr is 0.184 (7) under constraints, $g(\text{La})+g(\text{Sr})=1$, $x(\text{La})=x(\text{Sr})$, $y(\text{La})=y(\text{Sr})$, $z(\text{La})=z(\text{Sr})$, $B_{\text{iso}}(\text{La})=B_{\text{iso}}(\text{Sr})$.

$\text{La}_{0.8}\text{Sr}_{0.2}\text{CuO}_{2.5}$ is nearly the same as that of a superconductor $\text{La}_{1.8}\text{Sr}_{0.2}\text{CuO}_4$ in its normal state. However, no indications of a superconducting transition have been seen for any doping level examined. The Arrhenius plot, $\ln \rho$ against $1/T$, for $x=0$ gave a straight line at high temperatures, from the gradient of which an activation energy of 1,260 K (110 meV) was obtained. The temperature dependence for $0.05 \leq x \leq 0.15$ is rather weak: the resistivity gradually increases with decreasing temperature down to 5 K. Compare these results with the case of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$, where metallic behaviour appears for a much smaller hole concentration of $x \sim 0.01$ and superconductivity is observed for $x \geq 0.06$ (refs 18, 19). The difference may reflect the substantial difference in dimensionality between the two cuprates.

The temperature dependence of magnetic susceptibility (χ) is shown in Fig. 3b. For $\text{LaCuO}_{2.5}$ it decreases gradually with decreasing temperature to a minimum around 90 K, which is followed by a Curie-like increase at lower temperature. The data were fitted to the following equation,

$$\chi = \chi_0 + C/(T - \Theta) + \alpha T^{-1/2} \exp(-\Delta/T)$$

where χ_0 is a temperature-independent term, and the second term is a Curie–Weiss contribution ascribed to impurities and/or Cu^{2+} ions made free by crystal imperfections. The third term represents the bulk spin susceptibility expected for a two-chain, spin- $\frac{1}{2}$ Heisenberg antiferromagnetic ladder, where α is a constant factor and Δ is the magnitude of the spin gap²⁰. The fit was very good, as shown in Fig. 3b. The values obtained are $\chi_0 = 3.8$ (2) $\times 10^{-6}$ e.m.u./mol, $C = 1.42$ (1) $\times 10^{-3}$ e.m.u./mol, $\Theta = -5.0$ (1) K, $\alpha = 4.11$ (3) $\times 10^{-3}$, and $\Delta = 474$ (3) K. All these values are very similar to those reported for SrCu_2O_3 (ref. 10). Because χ_0 is near the sum of the temperature-independent contributions of the core diamagnetism (-6.2×10^{-5} e.m.u./mol) and the Van Vleck paramagnetism (6.0×10^{-5} e.m.u./mol)²¹, spin excitation is probably suppressed at low temperature. The fact that the magnitude of the spin gap is comparable to that for SrCu_2O_3 (420 K) suggests that inter-ladder interactions are weak as in SrCu_2O_3 , because such interactions would strongly reduce the magnitude of the spin gap³. The size of the free Cu^{2+} spins calculated from the Curie component is only 0.38%.

The doping dependence of magnetic susceptibility is also shown in Fig. 3b. The magnitude increases with doping, and, at

the same time, the characteristic decrease for $x=0$ due to the presence of the spin gap seems to fade gradually. If we assume identical temperature dependences for pure and doped samples and fit the data to the above equation, the magnitude of the gap is calculated to be 406 K ($x=0.025$) and 364 K ($x=0.05$), which is rather insensitive to doping. On the other hand, χ_0 increases rapidly with doping. This may correspond to the evolution of an in-gap state that is responsible for the increase in conductivity. The Curie constant or the number of free Cu ions increases only slightly.

It would be interesting to study the nature of the metallic state attained in the present system. If the one-dimensionality suggested from the crystal structure is real and remains intact upon doping, it is possible that a Tomonaga-Luttinger liquid is realized, which is a non-Fermi liquid predicted theoretically to appear in a correlated one-dimensional metal²². A further study using a single crystal is needed to confirm this possibility.

Unfortunately, the theoretical prediction of superconductivity in lightly doped ladders has not been confirmed in the material described here. It is possible that the superconducting correlation is suppressed by weak but non-negligible inter-ladder inter-

actions, or alternatively insufficient coupling between ladders prevents the three-dimensional superconducting phase transition, although the superconducting correlation survives within each ladder. Otherwise, if a hole-doped spin-ladder compound is essentially non-superconducting, it would raise an important question to be addressed theoretically. It would be a challenge for solid-state chemists and physicists to prepare new carrier-doped ladder systems with large chain numbers to study the effects of dimensional crossover. □

Received 16 June; accepted 7 August 1995.

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ACKNOWLEDGEMENTS. We thank Y. Kitaoka, H. Takagi and M. Ogata for helpful discussions, and Dr R. J. Cava for reminding us of some previous studies. This study was supported partly by a Grant-in-Aid for Scientific Research on Priority Areas given by the Ministry of Education, Science and Culture, Japan.

Continuous grey-scale image storage using optically aligned nematic liquid crystals

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LIQUID crystals have been widely used in flat-panel-display applications for many years. But attempts to use liquid crystals as optical data-storage media¹ have met with limited success, owing to both the difficulties of encoding grey-scale images² and the complexity of writing and rewriting schemes^{3,4}. Fundamental to all liquid-crystal-based optical devices is the control of average molecular orientation over macroscopic length scales, and it has recently been shown that polarized light can be used to induce such alignment with high spatial resolution^{5–12}. Here we show that a similar strategy can be used to record high-resolution, erasable, continuous grey-scale images. This approach shows potential for low-cost, high-density optical data-storage applications.

We have demonstrated previously how to create two states of alignment (0° and 90° twist) using optical alignment with polarized light^{5–9}. Typically, a cell comprising a glass substrate coated with an optically aligned azo dye/polyimide polymer is combined with another glass substrate, which is coated with a polyimide film and mechanically buffed (rubbed with fibrous cloth to induce alignment along the rub direction¹³), to form a cell

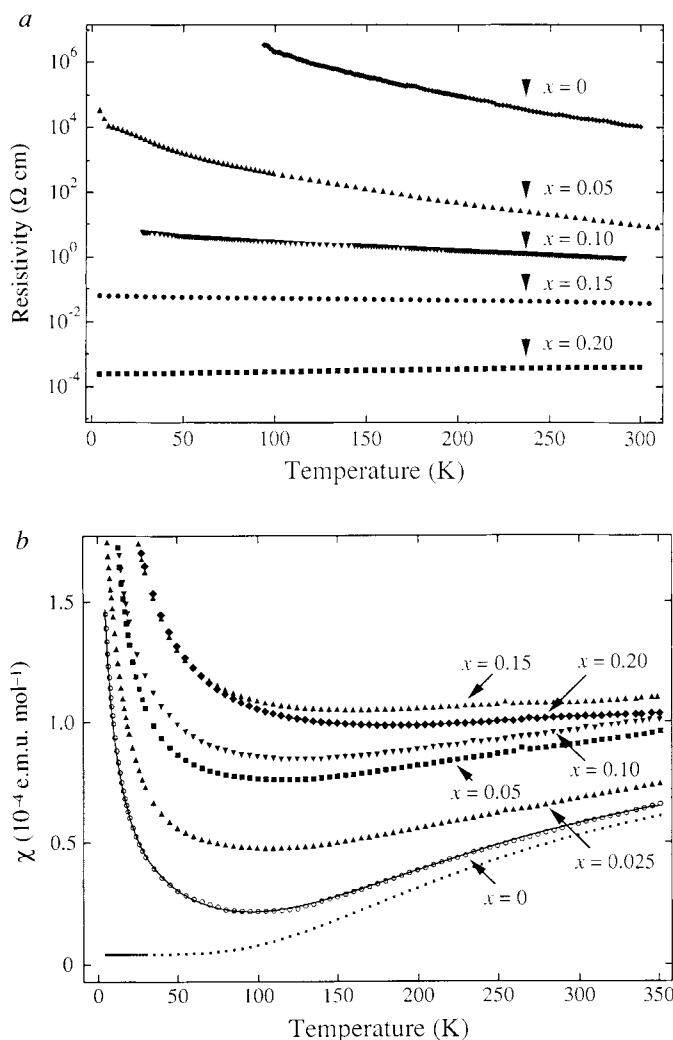


FIG. 3 *a*, A series of resistivity-temperature curves for $\text{La}_{1-x}\text{Sr}_x\text{CuO}_{2.5}$, measured by a standard four-probe method. *b*, Temperature dependence of magnetic susceptibility χ measured with a superconducting quantum interference device (SQUID) magnetometer at an applied magnetic field of 1 T on heating after zero-field cooling. The data for $x=0$ are a good fit to the equation in the text, as shown by the solid line. The lowest dotted line plots the calculated susceptibility for $x=0$ after subtraction of the Curie component.

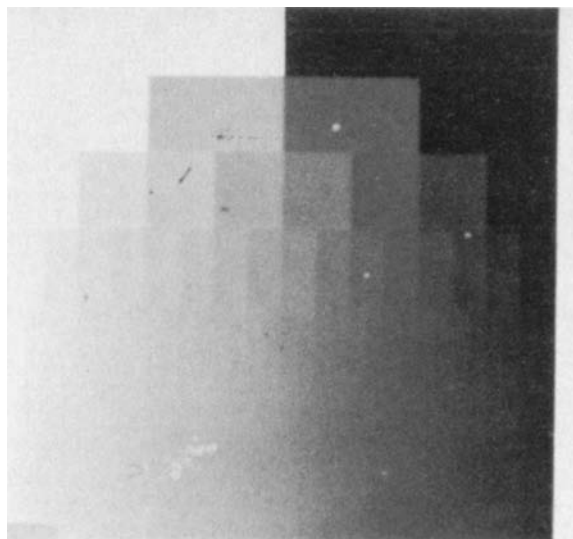


FIG. 1 Photograph of a cell with a grey-scale pattern between polarizers. In this case the azo dye in the polyimide was Disperse Orange 3 (Aldrich). The grey-scale pattern was written with the 514.5 nm line of an argon laser; with a beam waist of 35 μm and incident power of 0.4 W. The cell was translated at a constant velocity in the horizontal direction, while the polarization direction was varied with the modulator. After each row, the cell was moved one pixel width in the vertical direction.

with uniform liquid-crystal orientation along one direction. Because the mechanically rubbed polyimide film lacks azo chromophores, it is insensitive to the light used in the optical alignment process. A region of the cell is subsequently exposed to light polarized parallel to the original background alignment direction, which causes the liquid crystal adjacent to the azo dye/polyimide alignment layer to orientate perpendicular to the original alignment direction. As a consequence, in this region the liquid crystal takes on a twisted configuration, with the orientation direction rotating 90° through the cell.

To generate high-resolution grey-scale images in such a cell, we proposed focusing the light beam to a size comparable to the wavelength, and varying the twist angle of the liquid crystal by varying the polarization direction of the illuminating light. In the Mauguin limit¹³ ($\Delta n \times d \gg \lambda$, where Δn , d and λ are the birefringence of the liquid crystal, the thickness of the liquid crystal layer, and the wavelength of the light, respectively), the polarization of light passing through a twisted nematic region follows the direction of nematic orientation; that is, it rotates through the same angle. As a consequence, if the cell is between polarizers, the transmittance of each region varies with the twist angle. Thus, by controlling the polarization direction of the write beam, we can write pixels whose twist angle can take on any value between 0° and 90°. This means that the transmittance between polarizers, and hence the grey-scale of each pixel, can be varied continuously between the maximum and zero.

To demonstrate this concept, the azo-dye/polyimide-coated substrate was exposed to polarized light with wavelength of 514.5 nm from an argon laser to establish the initial alignment direction⁸. The polyimide-coated substrate was mechanically

buffed to induce uniform alignment when the cell was constructed. Cells 40–50 μm thick were assembled from the two coated substrates so that initial alignment directions were parallel, and were subsequently filled with EM Industries MLC6043-000 superfluorinated, ZLI1982 cyclohexyl, or E7 nematic liquid-crystal mixtures.

The images were written in the cells by mounting the cell on an X - Y translation stage so that the polarized write beam was normally incident on the azo-dye/polyimide-coated substrate side of the cell. The light from the argon laser passed through a polarization modulator, consisting of an electro-optical phase modulator and quarter-wave plate. The modulator varied the polarization of the beam in the X - Y plane from -45° to 45° relative to the vertical direction as a function of driving voltage. Computer-digitized 8-bit (256-level) grey-scale images were used to determine the twist angle of each pixel. The write beam polarization, writing duration, and cell translation were computer controlled.

Figure 1 shows a photograph of a grey-scale test pattern in a cell filled with the E7 nematic mixture as viewed between polarizers. From top to bottom, the number of grey-scale levels increases in powers of 2.

A photograph of a US dollar bill, written into a cell filled with MLC6043-000 liquid crystal, is shown in Fig. 2. The image resolution is 937×571 pixels with an 8-bit grey-scale. The image size in the cell was approximately 38 mm $\times$ 25 mm, with approximately 40 μm $\times$ 40 μm pixels.

Figure 3 demonstrates the generation of complementary images by rotating the output polarizer by 90°. A 702 $\times$ 738-pixel, 8-bit grey-scale computer image was used to write the

FIG. 2 Photograph of a cell with an image of a dollar bill between polarizers. The creases in the bill can be clearly seen. The azo dye from ref. 5 was used in the alignment layer. Exposure conditions were the same as for Fig. 1.



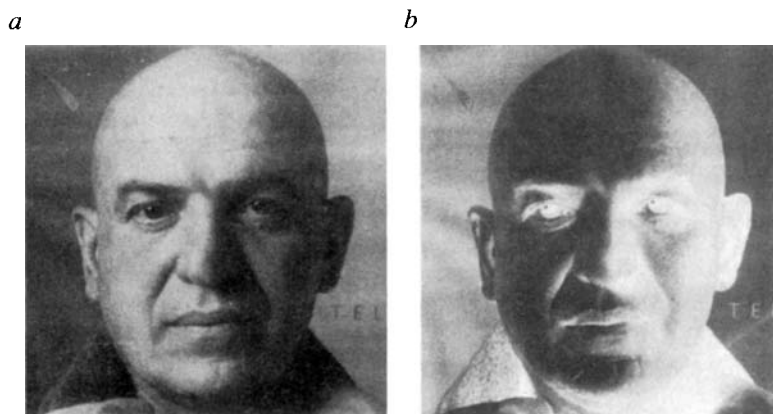


FIG. 3 Photograph of a cell between (a) polarizers and (b) polarizers with analyser rotated through 90° . The image was from a digitized photograph of Telly Savalas (Copyright 1978, Sid Avery). The alignment layers and the exposure were the same as for Fig. 2.

image in the cell filled with ZLI1982. Fig. 3a duplicates the original (positive) computer image, and Fig. 3b is the complementary (negative) image. Controlling the polarization of the outgoing light is a feature typically not present in other storage schemes, which could be useful, for example, in making a single photolithographic mask for positive and negative photoresists.

Under a polarizing microscope, the nematic orientation was seen to change continuously from one pixel to the next. There was a small number of visible liquid-crystal alignment defects between adjacent pixels and within each pixel. The images are therefore invisible to the eye without the aid of polarizers.

Previously, the polarized alignment of azo chromophores in liquid-crystalline polymers has been described as a potential medium for optical data storage^{3,4}. Azo chromophores distributed throughout the bulk of the polymer were aligned perpendicular to the polarization of the incident light. Possible mechanisms for the azo chromophore reorientation have been described elsewhere¹⁴. The orientated chromophores induced the orientation of the surrounding liquid-crystal polymer, creating an aligned domain in the polymer. The high viscosity of the polymer could reduce its usefulness as a storage medium.

In our work, a small optically induced change in a thin (on the order of $300 \text{ \AA}$ thick) layer of polymer doped with azo chromophores is amplified by the adjacent liquid crystal to create an aligned domain in the liquid-crystal medium (on the order of $1\text{--}100 \text{ }\mu\text{m}$ thick). The low absorption and reduced viscosity of the liquid crystal could result in improved performance for optical data-storage applications. Others have also shown that alignment polymers based on cinnamate esters can optically induce alignment of the adjacent liquid crystal by means of polarized ultraviolet radiation, and, therefore, demonstrated that the process is not limited to alignment polymers with azo chromophores¹⁰.

A short response time is desirable for optical storage media. The optically induced changes required for alignment of liquid crystals occur in the azo dye/polyimide film with minimum exposure times of $1 \text{ }\mu\text{s}$ (the lowest resolution of the experiment) or less⁸. This is comparable to that achievable with existing technologies¹. However, it is expected that the liquid crystal's response to the optically induced changes in the alignment layer will be slower. This could ultimately determine the minimum time between successive write and read cycles of a given pixel.

Optical energy density requirements for writing and erasure of this particular azo dye/polyimide film are on the order of 1 J cm^{-2} . Other authors have reduced, by changing the chemistry of the host polymer and the azo chromophores, the energy density requirements by a factor of 10 or more¹⁵. Fatigue of the azo chromophores occurs by photodegradation after many write cycles. Ultimately, it may be necessary to develop photosensitive alignment layers independent of azo chromophores to prevent fatigue. Because other photochemical mechanisms have been demonstrated to align liquid crystals, it is conceivable that suit-

able materials, less prone to fatigue, can be developed; for example, spiropyran monolayers have been shown to optically align liquid crystal materials¹⁶. In addition, spiropyrans have been extensively investigated for optical memory-storage applications with differing degrees of fatigue depending on the molecular structure and the surrounding polymer matrix¹⁷. Therefore, appropriate design of the photosensitive alignment polymer (for example, type of photochemistry, region of wavelength sensitivity and thermal properties) may reduce writing and erasure energy-density requirements as well as fatigue problems for optical data storage.

Manufacturing of high-quality liquid-crystal cells is a well established process used by liquid-crystal display manufacturers. Because only one read wavelength is usually required and the structure of the cell will be much simpler than that used in displays, optical data-storage media based on this technology have the potential to be low in cost. Increased storage capacities will further reduce the effective cost of this medium to below that of conventional media. IBM recently reported that current 3 Gbit in^{-2} storage densities were possible in their medium¹⁸. With the 8-bit grey-scale demonstrated in this paper and $1.3 \text{ }\mu\text{m}$ pixels (achievable with existing data-storage read/write heads¹), data storage densities of approximately 3 Gbit in^{-2} are possible with this liquid-crystal-based technology. The high birefringence of the liquid crystal and the high sensitivity of existing data-storage detection systems allow for the possibility of a 10-bit (1,024 levels) or higher grey-scale, which indicates even higher stored data densities than the current state of the art can be realized.

Further potential advantages of this technology over conventional media might be found by exploiting the electro-optical and alignment properties of liquid crystals. The orientation of the liquid crystal can also be controlled by applied electric and magnetic fields¹⁹. In particular, electric fields normal to the cell substrates created by electrodes can be used to align the liquid crystal perpendicular to the substrates. In this case, the information stored in the alignment polymer is no longer readable. Thus patterned electrodes can be used selectively to address particular regions of the medium to be read. This may be difficult to implement with traditional read geometries (that is, spinning media) but is conducive to parallel processing of the storage medium as is done in storage-display applications. By making both alignment layers of the cell optically sensitive to different wavelengths, other read/write schemes can be envisaged. For example, appropriate design of the medium can be used to increase storage capacity or to allow for logic operations between the information stored on each respective substrate (for example, separate addressing of each layer can be used to increase or decrease the twist angle of each domain).

We have demonstrated that optically controlled alignment allows the generation of continuous grey-scale images in liquid-crystal cells. The continuous grey-scale increases the stored infor-

mation density. Because the polarization state of the transmitted light can be accurately controlled, storage densities can be increased by one order of magnitude or more over traditional optical storage media based on binary pixels. The unique properties of this technology provide additional flexibility in the design of optical data-storage media and systems. □

Received 12 May; accepted 8 August 1995.

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ACKNOWLEDGEMENTS. Parts of this work were supported by the National Institute of Standards and Technology's Advanced Technology Program, the National Science Foundation's Science and Technology Center for Advanced Liquid Crystalline Optical Materials (ALCOM), and OTKA of Hungary. We thank S. C. Beard for technical support and S. T. Schelle for technical discussions.

Confirmation by X-ray diffraction of the endohedral nature of the metallofullerene Y@C₈₂

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THE synthesis of fullerenes encapsulating various metal atoms within the carbon cage (endohedral metallofullerenes) has stimulated wide interest^{1,2} because of their unusual structural and electronic properties. Most of the metallofullerenes prepared so far have been based on C₈₂, and have incorporated lanthanum^{1,3,5}, yttrium^{6,7}, scandium^{8,10} and most of the lanthanide elements^{11,12}. Although there has been some debate about the endohedral nature of these compounds^{2,13,14}, observations using scanning tunnelling microscopy^{15,16}, extended X-ray absorption fine structure^{17,18}, transmission electron microscopy¹⁹ and electron spin resonance^{3,6,8,10} have strongly suggested that the metal atoms are indeed inside the fullerene cages; theoretical calculations^{20,21} also indicate that this is the case. But until now, no structural model

has been derived experimentally to confirm the endohedral nature of the metallofullerenes. Here we report the results of a synchrotron X-ray powder diffraction study of Y@C₈₂ that confirms that the yttrium atom is located within the carbon cage. The yttrium atom is displaced from the centre of the C₈₂ molecule and is strongly bound to the carbon cage.

Soot containing Y@C₈₂ was produced in direct-current (500 A) spark mode under He flow at 50 Torr and collected under totally anaerobic conditions to avoid degradation of the metallofullerene during collection and handling^{15,16,22}. The Y@C₈₂ fullerene was separated and isolated from various hollow fullerenes (C₆₀–C₁₁₀) by two-stage high-performance liquid chromatography (HPLC)²². The isolation of Y@C₈₂ was confirmed by mass spectrometry for the corresponding HPLC fraction. The purity of the fullerene was >99.9%. Y@C₈₂ powder samples grown from toluene solvent²² were sealed in 0.3 mm int. diam. silica glass capillary. To obtain an X-ray powder pattern with good counting statistics, the Synchrotron Radiation X-ray powder experiment with Imaging Plates as detectors²³ was carried out at Photon Factory BL-6A₂. The exposure time was 1 hour. The wavelength of incident X-rays was 1.0 Å. The X-ray powder data for the hollow C₈₂ fullerene were also measured as a reference under the same experimental conditions. The X-ray powder pattern of Y@C₈₂ was obtained up to 2θ = 20°, which corresponds to 2.9 Å resolution. The resolution is enough to show whether the Y atom is in an endo- or exohedral position. Previous X-ray single-crystal and powder work²⁴ concluded that the space group of the toluene-C₈₂ crystal is P2₁ and determined preliminary structure parameters with a simplified structural model. The present powder pattern of the hollow C₈₂ shows very good agreement with the previous result²⁴. The powder pattern of Y@C₈₂ is also similar to that of hollow C₈₂. Therefore the

TABLE 1 Crystal data and structure parameters for Y@C₈₂ and C₈₂ with space group P2₁ obtained by Rietveld analysis

	Y@C ₈₂					C ₈₂				
	Site	x	y	z	Occupancy	Site	x	y	z	Occupancy
Radius of C ₈₂ (Å)										
Centre of C ₈₂ cage	2a	0.279 (1)	0.962 (6)	0.116 (4)	1.0	2a	0.2770 (3)	0.010 (1)	0.128 (2)	1.0
Y atom	2a	0.230 (2)	0.802 (3)	0.902 (3)	1.0					
Centre of C ₅ H ₅	2a	0.121 (1)	0.468 (5)	0.554 (5)	0.89 (2)	2a	0.096 (1)	0.416 (2)	0.565 (5)	1.0
Centre of C ₂ H ₃	2a	0.253 (5)	0.564 (8)	0.59 (1)	0.89 (2)	2a	0.235 (3)	0.439 (6)	0.532 (9)	1.0
Thermal parameter (Å ²) of:										
Y			14 (2)							
C ₈₂ cage			3 (3)					1.2 (9)		
Toluene			14 (3)					1 (7)		
R _i (%)			5.90					5.83		

space group is assigned as $P2_1$, which is monoclinic for both $Y@C_{82}$ and C_{82} .

The experimental data were analysed iteratively by a combination of Rietveld analysis^{25,26} and the maximum-entropy method (MEM)^{27,28}. It is well known²⁹ that the MEM can produce an electron-density distribution map from a set of X-ray structure factors without using any structural model and that the MEM map is consistent with the observed structure factors and least biased with respect to unobserved structure factors. In MEM analysis, any kind of deformation of electron densities is allowed as long as it satisfies the symmetry requirements.

First, Rietveld analysis was done using a preliminary reference model in which C_{82} molecules were described by a homogeneous spherical shell. Then the central position of the C_{82} sphere, the radius of the sphere, the position of the yttrium atom and the central position of the solvent toluene molecule were refined as structural parameters. The isotropic harmonic model was used for the temperature factors. The reliability factors (R -factors) based on the Bragg intensities, R_I , were 14.4% and 12.7% for $Y@C_{82}$ and C_{82} , respectively. The observed structure factors were evaluated by dividing the observed intensities at a data point according to the calculated contributions of the individual reflections by the modified Rietveld program. The number of

structure factors derived in the present analysis were 105 and 106 for $Y@C_{82}$ and C_{82} , respectively.

After the Rietveld analysis, the MEM analysis was done with a computer program, MEED³⁰, using $48 \times 32 \times 32$ pixels. In the MEM reconstruction, the structure factors were all treated independently as phased values. After the MEM analysis, the R -factors based on the structure factors, R_F , were as low as 1.5% and 1.4% for $Y@C_{82}$ and C_{82} , respectively. This preliminary analysis indicated that there exist local maxima on the C_{82} cage in $Y@C_{82}$ that could correspond to partial order due to the complicated rotation modes.

The MEM map provided much information for deriving a better structural model for $Y@C_{82}$. The simple spherical shell was no longer an appropriate model for the C_{82} cage and was modified to a combination of a spherical shell and eight pseudo-atoms. The toluene molecule was also remodelled by treating two parts, C_5H_5 and $C-CH_3$, separately, to express the directional feature along [201]. With this new structural model, the Rietveld and MEM analyses were repeated until no significant improvement was obtained. Eventually, the R_I factor improved from 14.4% to 5.9% ($R_{wp} = 3.0\%$). In Fig. 1a the best fit of the Rietveld analysis of the $Y@C_{82}$ is shown, and the fitting based on the MEM map is shown in Fig. 1b, for which the R_I

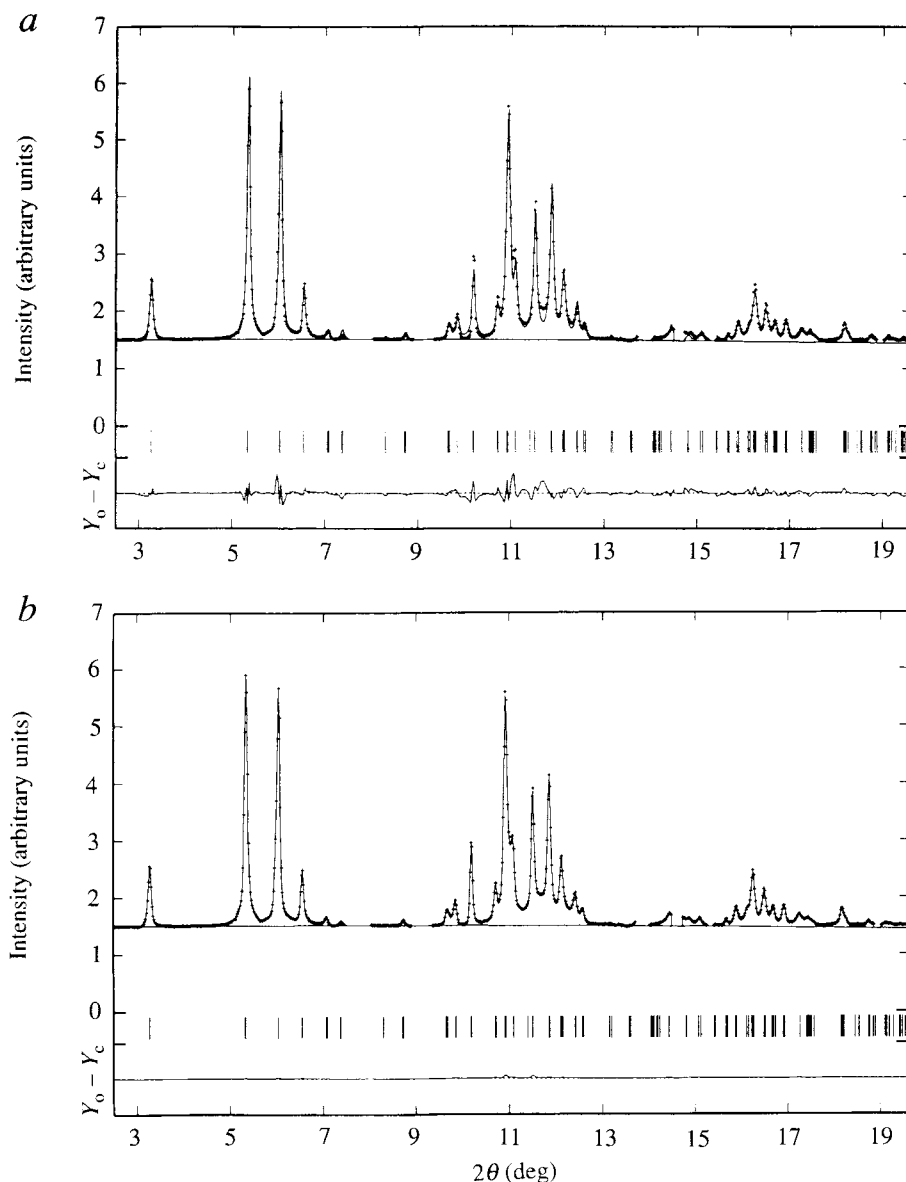


FIG. 1 a, The fitting result for $Y@C_{82}$; b, the fitting result for $Y@C_{82}$ based on the calculated intensities from the MEM electron density. The six weak impurity peaks are not included in the figures. In a, $R_I = 5.9\%$ and $R_{wp} = 3.1\%$; in b, $R_I = 1.4\%$ and $R_{wp} = 0.3\%$.

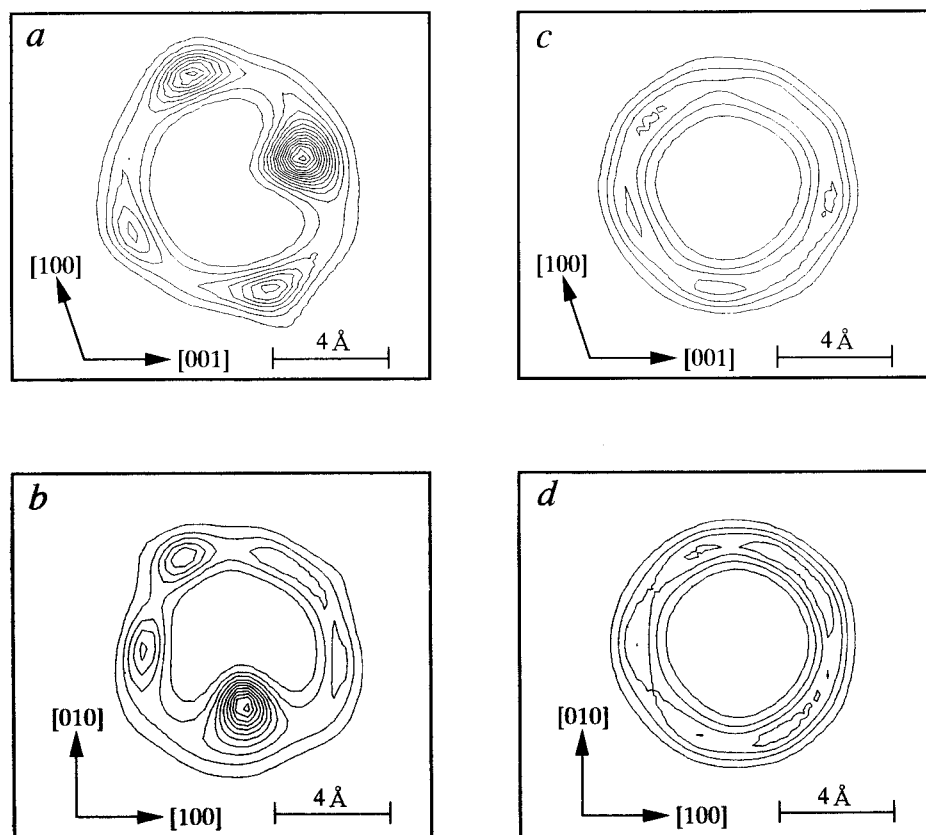


FIG. 2 The MEM electron density distributions of $Y@C_{82}$ for (a) the (010) and (b) the (001) sections, and those of C_{82} for (c) the (010) and (d) the (001) sections. The contour lines are drawn from 0.0 e Å^{-3} with 0.5 e Å^{-3} intervals. The endohedral nature of $Y@C_{82}$ is clearly shown in a and b.

factor is 1.4%. The final structure parameters of $Y@C_{82}$ and C_{82} determined by the Rietveld analysis are listed in Table 1. For the hollow C_{82} , only the toluene molecule was remodelled. The structure parameters of the hollow C_{82} fullerene in Table 1 are in good agreement with the previous results²⁴.

To visualize the endohedral nature of the $Y@C_{82}$, the MEM electron density distributions of $Y@C_{82}$ and C_{82} are shown in Fig. 2, which corresponds to sections of $Y@C_{82}$ and C_{82} . For the former there are remarkably high densities just inside the C_{82} cage (Fig. 2a, b), whereas there is no such high-density region in C_{82} (Fig. 2c, d). The number of electrons around the maximum is about 38, which is very close to the atomic number of an yttrium atom. Evidently, the density maximum at the interior of the C_{82} cage is the yttrium atom. The distance, calculated from the final Rietveld parameters, between the centre of the C_{82} cage and the Y atom, 3.14 Å, is shorter than the radius of the C_{82} cage, 4.10 Å. This also confirms that the Y atom is inside the C_{82} cage.

The cage structure of $Y@C_{82}$ differs from that of the hollow C_{82} fullerene. There are many local maxima along the cage in $Y@C_{82}$, whereas electron densities of the C_{82} cage are relatively uniform (Fig. 2c, d). This suggests that in $Y@C_{82}$ the rotation of the C_{82} cage is very restricted around a certain axis, whereas that of C_{82} is almost free. The MEM electron-density map further reveals that the yttrium atom does not reside in the centre of the C_{82} cage but is very close to the carbon cage, as suggested theoretically^{20,21}. The previous electron spin resonance^{6,7} and theoretical^{20,21} studies suggested the presence of a strong charge transfer interaction between the Y^{3+} ion and the C_{82}^{3-} cage, which may cause an aspherical electron density distribution of Y atoms. The Y–C distance calculated from the MEM map is 2.9 (3) Å, which is slightly longer than a theoretical prediction of 2.55–2.65 Å (ref. 21). Considering the limited resolution of the present study, higher-resolution diffraction studies should help to resolve this.

In the present MEM maps for both $Y@C_{82}$ and C_{82} , the solvent toluene molecules can be also recognized at almost the

midpoint of two C_{82} cages. The distances between the C_{82} cage and the central positions of the toluene molecules are slightly different in $Y@C_{82}$ and C_{82} . But these are not significant in the present endo- or exohedral determination for the metallofullerene.

The present study also reveals that the $Y@C_{82}$ molecules are aligned along the [001] direction in a 'head-to-tail' manner in the crystal, indicating the presence of strong dipole–dipole and charge-transfer interactions among the $Y@C_{82}$ fullerenes. The ability to achieve a molecular alignment of the endohedral metallofullerene in a certain direction in the crystal or on clean surfaces^{15,16} may in future lead to materials with novel solid-state properties. □

Received 6 March; accepted 3 August 1995.

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ACKNOWLEDGEMENTS. We thank N. Sakabe, A. Nakagawa, N. Watanabe, S. Adachi and S. Ikemizu for their experimental help at Photon Factory.

An Early Jurassic jumping frog

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WITH nearly 4,000 living species¹, frogs are numerically the most successful of modern amphibian groups. Their distinctive anatomy, which exhibits numerous unique features in both the axial and appendicular skeletons^{2–6}, represents a major departure from the body plan of Palaeozoic amphibians. We report here the discovery of the earliest known frog, associated with caecilians that retained limbs⁷, that exhibits primitive characters but shares with later anurans such features as fusion of the caudal vertebrae (urostyle), a rod-like ilium, and elongate hind limbs. The evolution of saltation in anurans entailed translocation of the ilium below the sacral rib, recruitment of the primitive tail musculature to transmit force from the hind limb to the axial skeleton during a jump, and development of iliosacral mobility. We reinterpret the caudopelvic transition from Palaeozoic amphibians to modern frogs with reference to *Triadobatrachus massinoti*, an Early Triassic amphibian that possesses some frog-like features⁸. The Early Jurassic age and Laurasian provenance of the new frog support the hypothesis⁴ that the widespread occurrence of primitive extant anurans reflects an ancient Pangaeian distribution.

Superorder Salientia Laurenti, 1768

Order Anura Rafinesque, 1815

Family Prosaliridae, new

Prosalirus bitis gen. et sp. nov.

Etymology. Latin, *Prosalire*, to leap forward; Navajo, *bitis*, high over it⁹.

Holotype. Museum of Northern Arizona (MNA) V 8725 (Fig. 1), associated remains of at least two individuals: frontoparietal, stapes, sphenethmoid, parasphenoid, maxilla, premaxilla, angulosplenial, 6 presacral vertebrae, ribs, 3 coracoids, clavicle, 3 humeri, radioulna, carpalia, metacarpalia, 2 partial ilia, tibiofibula.

Referred material. Museum of Comparative Zoology (MCZ) 9324 A (Fig. 2), associated remains of at least two individuals: parasphenoid, 2 cervical (atlas) vertebrae, 1 sacral and 8 presacral vertebrae, urostyle, scapula, coracoid, radioulna, 6 carpalia, 4 metacarpalia, phalanges, 2 ilia; MCZ 9323 A, coracoid, femur, tibiofibula, and proximal tarsals (one incomplete).

Horizon. Silty facies of the Kayenta Formation.

Locality. Gold Spring quarry (35°45.785' N, 111°05.382' W), Adeii Eechii Cliffs, Coconino County, Arizona, USA.

Age. Early Jurassic (Pliensbachian).

Diagnosis. Derived feature shared with other lissamphibians¹⁰: pedicellate teeth. Derived features shared with other salientians (which includes anurans and *Triadobatrachus massinoti*)⁵ are: uncapitate and shortened ribs (Fig. 1), and an elongate and anteriorly directed iliac blade (Fig. 2b). Derived characteristics shared with other anurans (*sensu*⁵) include: ilia articulate ventral to sacral diapophysis¹¹, radioulna (fused radius and ulna; Fig. 1), hind limb substantially longer than forelimb (Table 1), tibiofibula (fused tibia and fibula), elongate proximal tarsus (Table 1), and caudal vertebrae synostosed to form an elongate, hollow urostyle (Fig. 2c). Distinguished from other basal anurans by a combination of primitive and derived features. Unfused tibiale and fibulare (fused in *Leiopelmatids*). Roughly 60 maxillary teeth (<20 in *Vieraella herbsti*)². A well ossified sphenethmoid with a moderately broad, bony nasal septum and an ossified orbito-nasal canal (unossified in *Notobatrachus degiustoi*², *Ascapus truei*, *Leiopelma* spp.). The sphenethmoid ossification extends posteriorly to include the anterior margin of the optic foramen (cartilaginous in *Leiopelma* and discoglossids). The ilium is primitive in lacking a dorsal crest (present in *Eodiscoglossus oxoniensis*¹²) and supra-acetabular fossa (present in *E. oxoniensis* and *Eodiscoglossus santonjae*¹²), and in possessing only a slight rugosity in place of a dorsal tubercle (tubercle distinct in *E. oxoniensis*¹², prominent in *E. santonjae*¹² and *Enneabatrachus hechti*¹³). The stapes (Fig. 1) is structurally similar to that in modern anurans (such as *Hyla albomarginata*¹⁴; the stapes is lost in *A. truei* and *Leiopelma* spp.). The atlas bears a large, funnel-shaped notochordal fossa between the cotyles (comparable in size to that in *E. oxoniensis*¹²; such fossae are usually absent in Recent taxa, with the exception of *Leiopelma* spp.^{15,16}

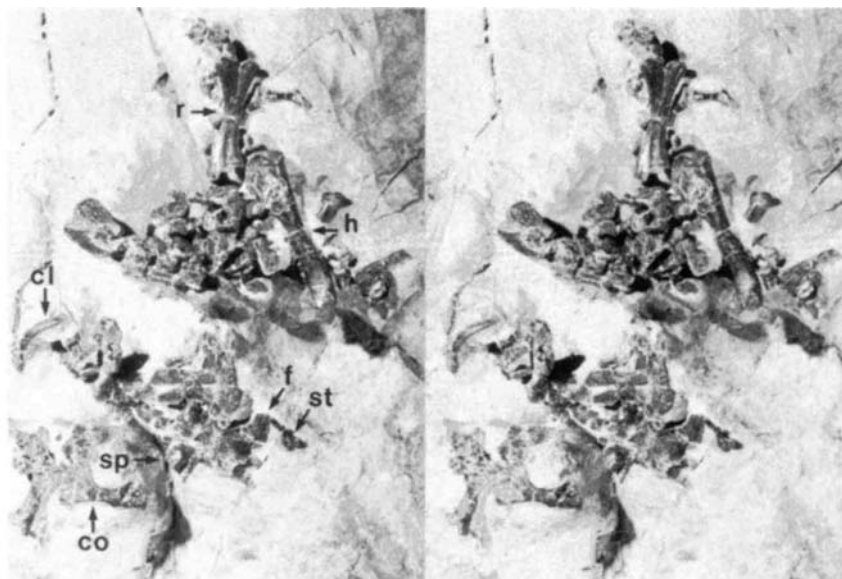


FIG. 1 Stereoscopic photographs of associated cranial and postcranial bones of *Prosalirus bitis*, gen. et sp. nov. (MNA V 8725; $\times 2.1$): cl, clavicle; co, coracoid; f, frontoparietal, arrow points to posterolateral margin; h, humerus; to the left of the humerus lie five vertebrae, one of which bears a rib with an uncinat process; r, radioulna; sp, sphenethmoid (ventral aspect), arrow points to rostral end; above the sphenethmoid lies a parasphenoid; st, stapes.

TABLE 1 Relative lengths of hindlimb segments in Mesozoic and Recent anurans, *Triadobatrachus massinoti* and various Palaeozoic amphibians

	N	Femur: dorsal vertebra	Tibiofibula: femur	Proximal tarsus: femur	Hum. + radioulna: fem. + tibiofibula
Palaeozoic amphibians					
<i>Proterogyrinus scheelei</i>	1	5.0	0.65		0.85
<i>Amphibamus lyelli</i>	1	6.3	0.59		0.82
<i>Apateon pedestris</i>	1	2.9	0.56		0.83
Early Triassic salientian					
<i>Triadobatrachus massinoti</i>	1	4.9	0.65	0.22	0.78
Mesozoic Anura					
<i>Prosailirus bitis</i> (Early Jurassic)		9.9	1.01	0.53	0.60
<i>Notobatrachus degiustoi</i> (Middle Jurassic)	1	8.5	0.94	0.40	0.73
<i>Eodiscoglossus santoniae</i> (Early Cretaceous)	1	9.6	1.0	0.53	0.50
Recent Anura					
<i>Ascaphus truei</i>	20	9.8 ± 0.6	1.1 ± 0.05	0.69 ± 0.04	0.58 ± 0.04
<i>Leiopelma</i> spp.	4	12.6 ± 1.1	1.1 ± 0.04	0.65 ± 0.04	0.53 ± 0.05
<i>Bombina orientalis</i>	6	8.1 ± 0.7	1.0 ± 0.01	0.66 ± 0.01	0.56 ± 0.01

Ratios of femoral length are expressed in terms of the average length of a dorsal vertebral segment, rather than skull or body lengths, which are more variable. Data are from specimens in the Museum of Comparative Zoology except *Proterogyrinus scheelei*²¹, *Amphibamus lyelli*²², *Triadobatrachus massinoti*⁸, *Notobatrachus degiustoi*² and *Eodiscoglossus santoniae*². Data on *Prosailirus bitis* are taken from associated hindlimb elements (MCZ 9323 A) and an average of the lengths of five vertebrae (MCZ 9324 A). In Palaeozoic amphibians tibial and fibular lengths differ, and accordingly average values were used to calculate ratios.

in which they are relatively small), the vertebrae are notochordal, and the sacral-urostyle articulation is cartilaginous (non-condylar). Four (possibly five) pairs of ribs, one of which is ankylosed, the others joined by mobile joints (*V. herbsti* has three mobile ribs, and *N. degiustoi* four to five mobile ribs³). Uncinate processes project anteriorly on two ribs, and a third rib is trifurcate distally.

Central to the question of the origin of anuran saltation is the major transformation of the iliosacral joint from the primitive amphibian condition in which the ilium contacts the dorsal (or lateral) surface of the sacral rib (Fig. 3*b*) to the uniquely anuran articulation of the ilium with the ventral (anatomically medial) surface of the sacral diapophysis (Fig. 3*a, d*). Furthermore, modern anurans have abandoned iliosacral rigidity which in most other tetrapods is required for force transmission between hind limb and axial skeleton. Various configurations of sacral diapophyses and ligaments allow rotational and translational

movements¹¹ which facilitate reorientation of the trunk during the initial phase of a jump and are also used in walking, swimming and burrowing. Anuran saltation also uses an unusual mechanism of force transmission between hind limbs and axial skeleton. With the caudal vertebrae consolidated into a rod-like urostyle, caudopelvic muscles transmit thrust from the ilium to the sacrum through the urostyle (for example, coccygeosacralis¹⁷) and lever the urostyle and presacral vertebral column during the initial phase of a jump¹⁸. Iliosacral mobility is possible in anurans because the urostyle and caudopelvic muscles have in part supplanted the role of the iliosacral joint in force transmission.

Evidence for the development of these distinctively anuran features is represented by *Triadobatrachus massinoti*. Rage and Roček⁸ concluded that this form was not a 'true and efficient jumper' because the limbs exhibit few 'incipient' saltatorial features (elongation of the ilia and proximal tarsals). Although

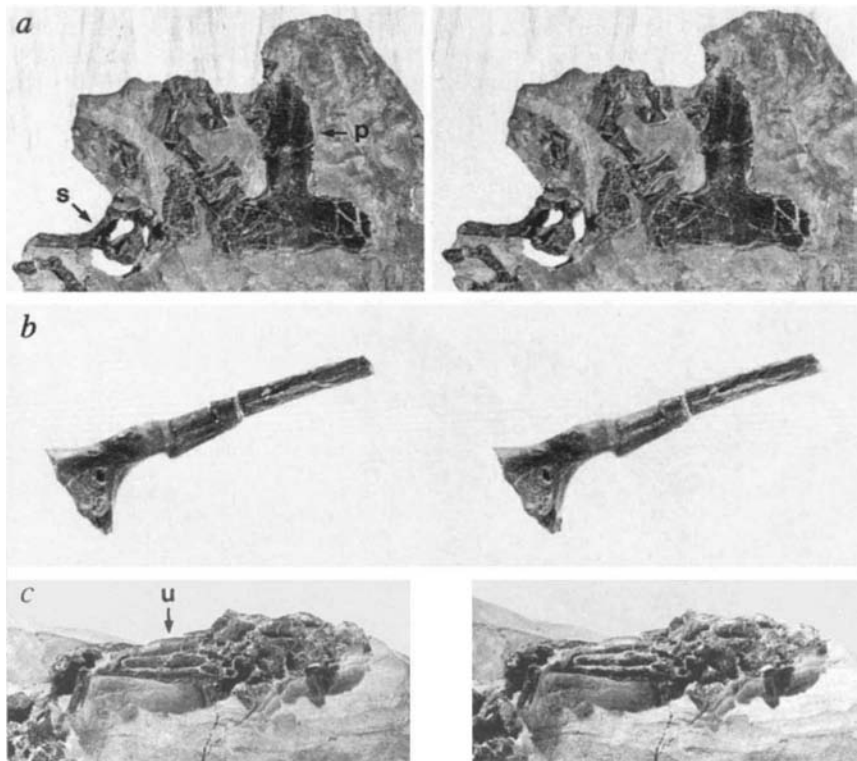


FIG. 2 Stereoscopic photographs of *Prosailirus bitis*, gen. et sp. nov. (MCZ 9324 A). *a*, Parasphenoid (p), carpalia and a sacral vertebra (s), ×2.8. The sacral vertebra (shown in posterior view) lacks postzygapophyses. Each sacral diapophysis is roughly triangular in cross-section and is flat ventrally for contact with the ilium. Emerson¹¹ classified modern anuran iliosacral joints on the basis of ligament configuration and presence/absence of ligamentous scars on the diapophyses. The shape of the diapophyses in *P. bitis* most closely conforms to Emerson's Type IIB (present in *Ascaphus*, most ranids, and some leptodactylids¹¹), but as the distal ends are not preserved a determination of the presence of ligament scars is not possible. *b*, Right ilium (lateral view), ×3.6. *c*, Urostyle (u) associated with crushed sacral and presacral vertebrae, ×3.9.

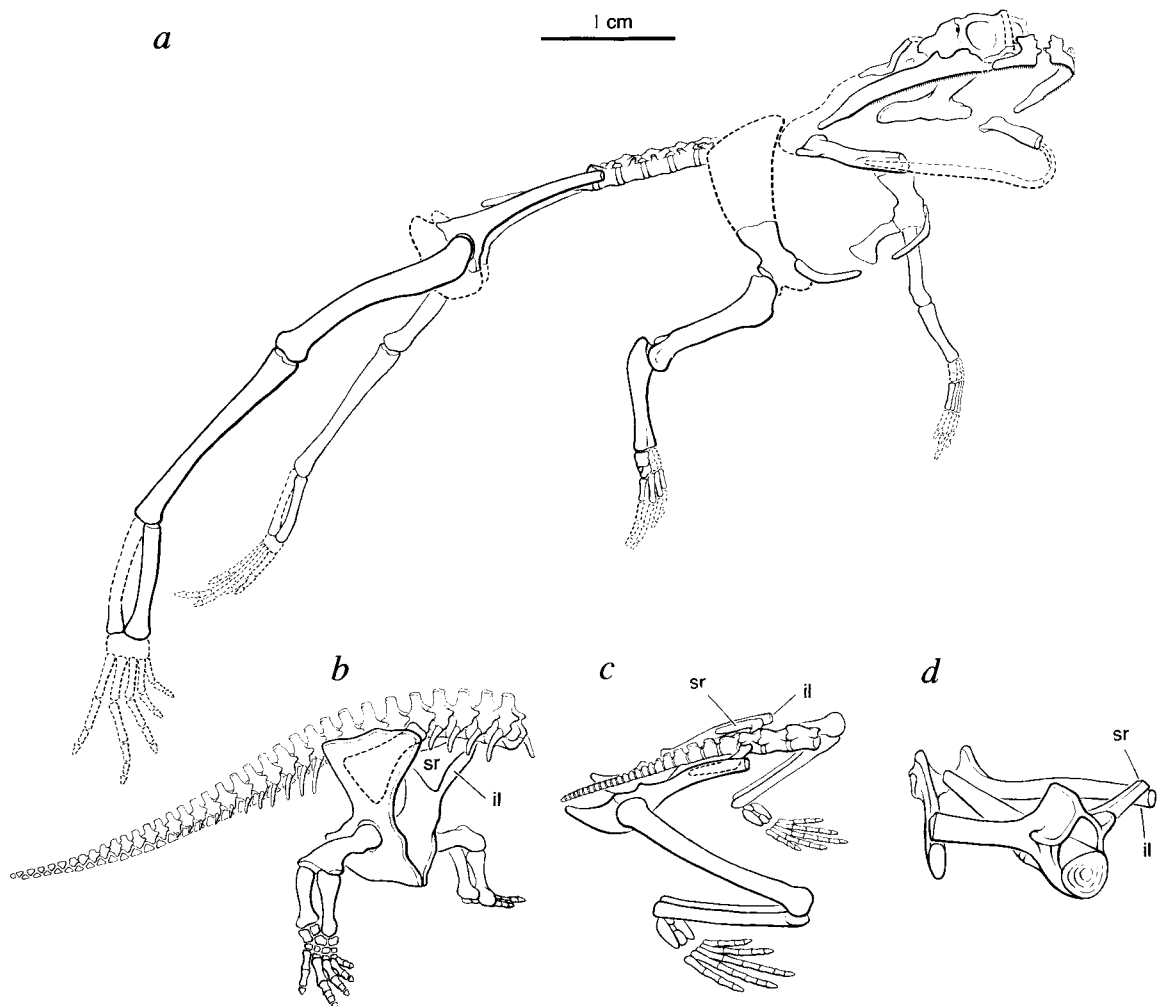


FIG. 3 *a*, Reconstruction of *Prosalirus bitis*, gen. et sp. nov., based on MNA V 8725, MCZ 9323 A and MCZ 9324 A. Before the discovery of *Prosalirus bitis*, the earliest known anuran was *Vieraella herbsti*², but the age of this specimen has not been definitively determined (?Middle Jurassic; Toarcian–Aalenian²³), the pelvis is unknown and hind limbs are incomplete. In *P. bitis*, the relative elongation of the femur, tibiofibula and proximal tarsus (Table 1), the prolongation of the ilium and the presence of a rod-like urostyle are features unequivocally indicative of saltatorial locomotion. Major modifications of the anuran pelvis for saltation (urostyle, iliac contact with the ventral surface of the sacral diapophysis, and iliosacral mobility) thus appear to have been established by Early Jurassic (Pliensbachian) time. Subsequent modifications involved further loss of true ribs (and presumably truncal shortening through reduction in presacral vertebral number), elongation of distal limb elements, and fusion of the tibiale-fibulare. *b–d*, Major stages in the evolution of the anuran pelvis (not to scale). *b*, Reconstruction (anterolateral view) of the sacral region of the Palaeozoic amphibian *Dissorophus multicinctus* (based on MCZ 4175) representing the

primitive tetrapod pattern of iliosacral joint relationships. The iliosacral joint is formed by the relatively broad, vertically directed ilium (il) that articulates with the lateral surface of the sacral rib (sr). *c*, Reconstruction of the sacral region in *Triadobatrachus massinoti* (dorsolateral view, modified from ref. 8). The ilium forms a narrow, anterodorsally directed process, and the acetabulum is positioned behind (rather than below, as in *b*) the iliosacral joint. The alignment of the long axis of the iliosacral articulation with the vertebral column is mechanically suited to transmit forces during hindlimb propelled jumping. The dorsoventral narrowing of the iliosacral contact in *T. massinoti* represents an intermediate stage in the translocation of the ilium from a primitive position on the anatomically dorsal surface of the sacral rib (*b*) to the ventral surface of the diapophysis (*d*). *d*, Reconstruction (anterolateral view) of the iliosacral joint in *P. bitis* in which the ilium lies ventral to the sacral diapophysis. Positioning of the ilium below the diapophysis in anurans establishes a fulcrum about which the vertebral column rotates during the initial stage of a jump and transmits upwardly directed forces from the hindlimbs through the ilium to the vertebral column.

T. massinoti lacks the extreme truncal shortening and hindlimb elongation associated with anuran saltation, the reconfiguration of the pelvis and shortening of the tail (Fig. 3*c*) represents an early stage in the evolution of the anuran muscular linkage between the urostyle and ilium. The narrow, anteroposteriorly aligned iliosacral joint of *T. massinoti* (Fig. 3*c*) is a structural intermediate in the translocation of the iliosacral contact from the dorsal (lateral) surface of the sacral rib (Fig. 3*b*) to the ventral (medial) surface of the sacral diapophysis (Fig. 3*d*). However, the extensive, congruent contact between the elongate iliac blade and sacral rib exhibited by *T. massinoti* is indicative of a firm rather than a mobile union; the evolution of the anuran caudopelvic linkage therefore appears to have preceded the development of iliosacral mobility. In *Prosalirus bitis* the very

slender anterior ends of the ilia (Fig. 2*b*) and rod-like sacral diapophyses (Fig. 2*a*) are evidence of a restricted iliosacral contact (Fig. 3*d*) and mobility.

The earliest fossil records of salamanders¹⁹, caecilians⁷ and anurans are all now known to lie in Laurasian landmasses. These new data contrast with previous views on the early biogeographic history of caecilians and frogs. As in the case of caecilians, the early biogeographic history of anurans had been thought to be centred in Gondwana²⁰; basal fossil anurans, *Notobatrachus* (Late Jurassic, South America) and *Vieraella* (Middle Jurassic, South America), share a Gondwanan distribution with *Triadobatrachus*. The geological ages and occurrences of *Triadobatrachus* (Early Triassic, Madagascar) and *Prosalirus* (Early Jurassic, North America) are evidence that anurans

differentiated and attained a Pangaeian distribution by the Early Jurassic, supporting the hypothesis that the cosmopolitan distribution of extant primitive frogs (*Ascaphus*, North America; *Leiopelma*, New Zealand) reflects this ancient distribution⁴ rather than more recent invasions of different landmasses. □

Received 3 May; accepted 13 July 1995.

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ACKNOWLEDGEMENTS. We thank W. W. Amaral for specimen preparation, A. H. Coleman for stereophotography, L. L. Meszoly for rendering Fig. 3, J. P. O. Rosado for herpetological materials in the Museum of Comparative Zoology, W. R. Downs for undertaking a more accurate (GPS) determination of the coordinates of the Gold Spring quarry than those previously published, S. Madsen and W. R. Downs for field collection of the Keyenta frogs and A. M. Baez, J. E. Cadie, D. C. Cannatella, S. B. Emerson, A. R. Milner and L. Trueb for critical advice. Supported by grants from NSF, the National Geographic Society and the Research Foundation of the University of Pennsylvania.

Patterning of the *Caenorhabditis elegans* head region by the *Pax-6* family member *vab-3*

Andrew D. Chisholm & H. Robert Horvitz

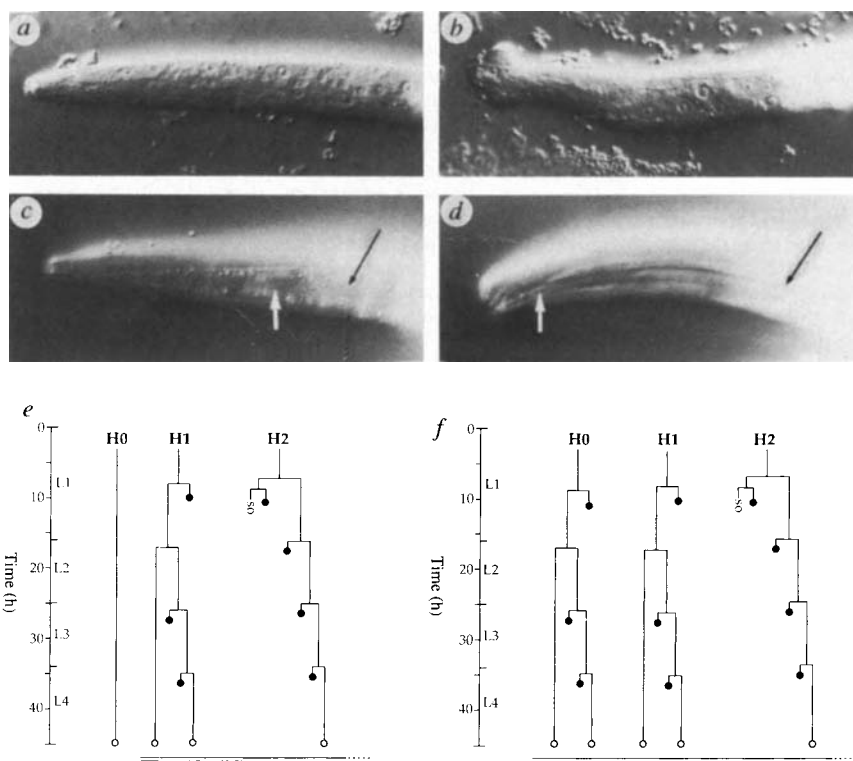
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THE *Pax-6* genes are important for eye development in both vertebrates and *Drosophila*. Mutations in the human *PAX6* gene are found in patients with a variety of eye disorders, including aniridia and Peters' anomaly^{1–3}, and mutations in the *Drosophila Pax-6* homologue cause the *eyeless* phenotype⁴. In the nematode *Caeno-*

***rhabditis elegans*, *vab-3* mutants display many defects in head-region development, including aberrant morphogenesis, transformation of hypodermal (epidermal-like) cell fates to those of posterior homologues, and abnormal specification of neurons. Here we show that *vab-3* is a member of the paired-domain-containing *Pax-6* gene family and is expressed in head-region cells. This *C. elegans Pax-6* locus can also encode proteins lacking the paired domain⁵. Our results suggest that a primordial role of the *Pax-6* gene family could have been to pattern part of the head region, and that *Pax-6* genes subsequently evolved to be more specifically involved in eye development.**

C. elegans vab-3 mutants were first isolated because of their gross abnormalities in head morphogenesis, resulting from defects in the embryonic migration or determination of head hypodermal cells (Fig. 1a, b)⁶ (A.D.C. and H.R.H., in preparation). For all nine *vab-3* alleles the expressivity of the head morphogenesis defect is variable, hence the gene name *vab* (vari-

FIG. 1 *vab-3* mutants have defects in head development. a–d, Nomarski photomicrographs of head regions of the wild-type and *vab-3* mutants. Left lateral views, anterior to the left, dorsal up. a, Head region of a wild-type L1 larva. b, Head region of a *vab-3(e648)* mutant L1 larva. In *vab-3* mutants the head morphology ranges from almost wild type to so extremely abnormal that the animals arrest development as embryos or larvae with grossly deformed heads. c, d, Left lateral views of head region of c, wild-type and d, *vab-3(e648)* mutant adults. The position of the deirid sensillum is marked by the black arrow, and the most anterior extent of the alae is marked by the white arrow. In the wild-type adult, alae extend just anteriorly to the deirid, whereas in *vab-3* alae extend to the tip of the head. e, Wild-type head cell lineages (hours after hatching at 20 °C). The head lateral hypodermal cells H0, H1 and H2 execute different lineages⁷. In the L4 stage the H-derived seam cells H1.aa, H1.appp and H2.pppp make lateral alae (shown as three lines), but the most anterior seam cell H0 does not make alae. f, Head cell lineages of *vab-3* mutants. Using Nomarski optics⁷ we followed the complete lineages of the H cells on both left and right sides in two *vab-3(e648)* animals and one *vab-3(sy66)* animal, and partial lineages in several other animals. In all three animals at least one of the two H0 cells divided in a pattern identical to that of wild-type H1, generating two seam cells that made alae (open circles) and three hypodermal nuclei (filled circles); the other H0 cells divided in patterns similar to that of H1 but with different orientations of their division axes, and in one case with an extra division during the L2 stage.



able abnormal). Head neuron fates are transformed in *vab-3* mutants, as judged by ultrastructural criteria⁶. We found that *vab-3* mutants also show defects in the determination of the larval hypodermis. In wild-type first-stage larvae the lateral hypodermis or seam is composed of ten cells, these being H0, H1, H2, V1–6 and T, of which all except H0 are blast cells, generating seam cells, hypodermal cells and neurons⁷. Seam cells derived from H1, H2, V1–6 and T make adult cuticle-bearing ridges known as alae. H0, the anteriormost seam cell, does not divide and makes adult cuticle that lacks alae; thus, in the wild type, alae extend anteriorly to the level of the isthmus of the pharynx (Fig. 1c); in *vab-3* mutants, alae extended further anter-

FIG. 2 Cloning of *vab-3*. **a**, Genetic mapping placed *vab-3* close to and to the left of the previously cloned gene *daf-12* (W.-H. Yeh and D. Riddle, personal communication), and within 0.015 map units of *mab-18* (ref. 5) (A.D.C. and H.R.H., in preparation). Cosmid clones and subclones were tested for rescue of *Vab-3* phenotypes as described below; the fraction of independently derived transformed *F*₂ populations that showed rescue is given for each clone. From comparison of cDNA and genomic DNA sequences (genomic sequence not shown) we determined that *vab-3* cDNAs derive from 12 exons, labelled 1–7 and 10–14; the *mab-18*-specific exons 8 and 9 are located between exons 7 and 10 (ref. 5) (not shown). ATG and TAG indicate the predicted start and stop sites of translation, respectively. Exons encoding the paired domain and homeodomain are shown as shaded boxes. Restriction enzyme abbreviations: B, *Bam*HI; N, *Nhe*I; Sc, *Sac*I; S, *Sal*I. **b**, Nucleotide and predicted amino-acid sequences (single-letter code) of *vab-3* cDNAs. Nucleotides are numbered beginning at the first nucleotide of cDNA clone 15c (see below); the sequence of cDNA clone B6 begins at position 255. There are two possible initiator ATG codons (shown in bold); the second ATG is likely to be the authentic start of translation as it matches the consensus (A/C)A(a/c)(A/C)ATG for initiation in *C. elegans* (M. D. Perry, G. H. Hertz and W. B. Wood, personal communication), whereas the upstream ATG context does not match the consensus; amino acids are therefore numbered in italics from the second ATG. There is an in-frame stop codon two codons 5' to the first ATG. Splice sites, shown as vertical lines, were determined by comparison with the genomic DNA sequence. The paired domain (residues 6–132) and homeodomain (residues 217–276) are shown in bold. The region of VAB-3 C-terminal to the homeodomain is rich in proline, serine and threonine residues (36% P+S+T from residues 277 to 455), like those of other *Pax-6* family members. The poly(A) tail is preceded by a consensus polyadenylation sequence (underlined) (T. Blumenthal, O. White and C. Fields, personal communication).

METHODS. *mab-18(bx23)* had been rescued by cosmids from the region of the *C. elegans* physical map to the left of *daf-12* (ref. 5). We used standard techniques²¹ to transform animals of genotype *him-8(e1489); vab-3(e648)* with DNA from cosmids in the *mab-18* region and with the plasmid pRF4, which confers a dominant Roller phenotype²¹ and can be used as a marker for transgenic animals. We established stable transgenic lines for each clone tested and scored the *Vab-3* phenotype in transgenic animals. Animals transgenic for the cosmid C56D7 showed complete rescue of the male tail and other defects of *vab-3*. We subcloned fragments of C56D7 and tested them for rescue of the *Vab-3* phenotype in stable transgenic lines. We used subclones of the 22-kb *Sal*I–*Nhe*I minimal rescuing fragment as probes to screen 3.6×10^6 plaques of a λ ZAPII cDNA library constructed from mixed-stage poly(A)⁺RNA²² and isolated two positive clones, with inserts of 2.3 and 1.7 kb. We determined the sequences of both strands of the 2.3-kb cDNA (clone B6) using the ABI PRISM cycle sequencing kit and an ABI 373A automated sequencer. We determined the sequences of the ends of the 1.7-kb insert and found that it corresponded to the 3' 1.7 kb of the longer clone. We also screened 6×10^5 plaques of a λ gt11 cDNA library made from embryonic RNA²³ and identified approximately 500 positive clones, of which we analysed 24. By hybridization with genomic DNA fragments and the 1.7-kb cDNA insert we determined that 17 of the 24 clones contained both the paired domain and the same 3' end as the B6 clone. Using polymerase chain reaction (PCR) and primers complementary to the vector and the paired domain, we amplified the 5' ends of the cDNA clones, cloned the PCR products and determined their sequences. The longest clone (15c) extended 255 bp further 5' than the B6 2.3-kb insert; all other clones analysed had slightly shorter 5' ends.

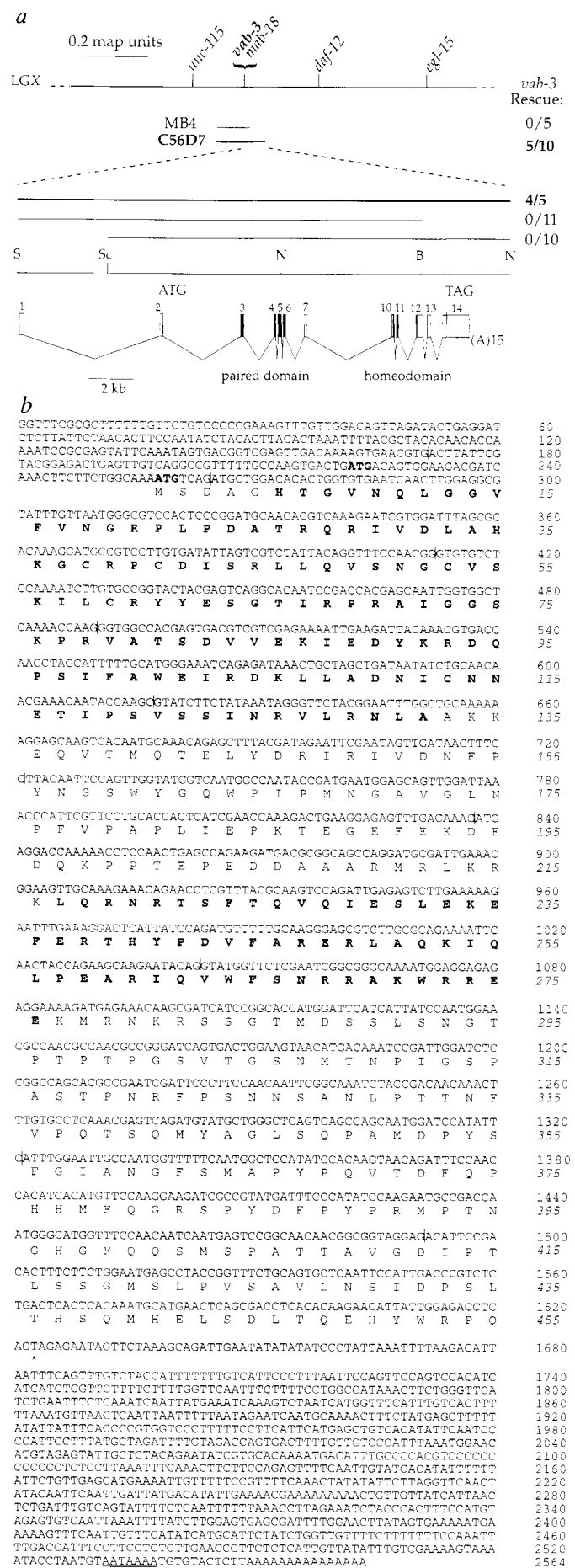


FIG. 3 Comparison of sequences of paired domains and homeodomains of the PAX-6 protein family and locations of *vab-3* mutations in the paired domain. The VAB-3 paired domain and homeodomain are aligned with the sequences of the paired domains and homeodomains of human PAX6 (ref. 24), zebrafish Pax-6 (ref. 13), *Drosophila eyeless*⁴ and sea urchin Pax-6 (ref. 25). The fraction of identities to VAB-3 within each domain is shown. The mouse and quail Pax-6 proteins are identical to the human PAX6 protein in these domains^{9,26}. Residues invariant among all previously known paired domains and among all paired-class and paired-like homeodomains²⁷ are marked by asterisks. The *vab-3* mutant e648 has a GC-to-AT transition converting tryptophan 101 (TGG) to an opal stop codon (TGA); sy66 has an AT-to-TA transversion converting isoleucine 103 (ATC) to asparagine (AAC); and sy281 has a GC-to-AT transition converting glycine 19 (GGG) to arginine (AGG). **METHODS.** Genomic DNAs from strains carrying four *vab-3* alleles (e41, e648, sy66 and sy281) were amplified using PCR and primers flanking the exons that encode the paired domain. Preparation of genomic DNA,

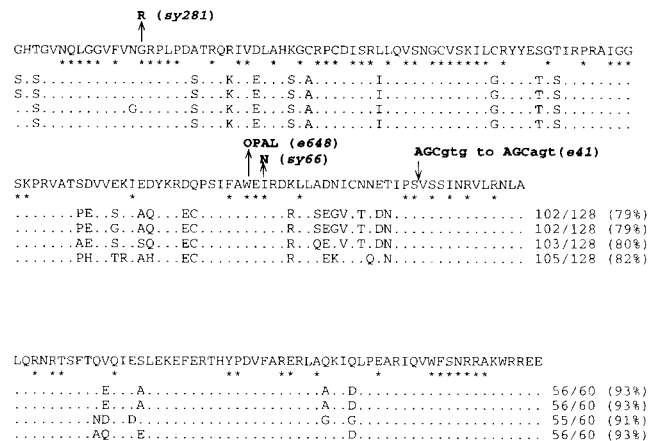
Paired domains

VAB-3
Human PAX6
Zebrafish Pax-6
Drosophila eyeless
Sea urchin Pax-6

VAB-3
Human PAX6
Zebrafish Pax-6
Drosophila eyeless
Sea urchin Pax-6

Homeodomains

VAB-3
Human PAX6
Zebrafish Pax-6
Drosophila eyeless
Sea urchin Pax-6



PCR and determination of sequences were as described²⁸. Sequences of the primers used are available upon request. Database searches were done at the National Center for Biotechnology Information using the BLAST network service.

iorly (Fig. 1d). By cell lineage analysis we found that this phenotype results from a transformation of the fate of H0: in *vab-3* mutants H0 divides in a pattern identical to that of the wild-type H1 cell, generating hypodermal nuclei and two alae-producing seam cells (Fig. 1e,f). The *vab-3* alleles cause a recessive phenotype and act as loss-of-function mutations (A.D.C. and H.R.H., in preparation), suggesting that, in the wild type, *vab-3* specifies many diverse cell fates in the head region, including that of the most anterior lateral hypodermal cell H0. In addition, *vab-3* mutants display defects in gonadogenesis and in the male tail (A.D.C. and H.R.H., in preparation; see ref. 8), suggesting that *vab-3* functions in multiple developmental processes.

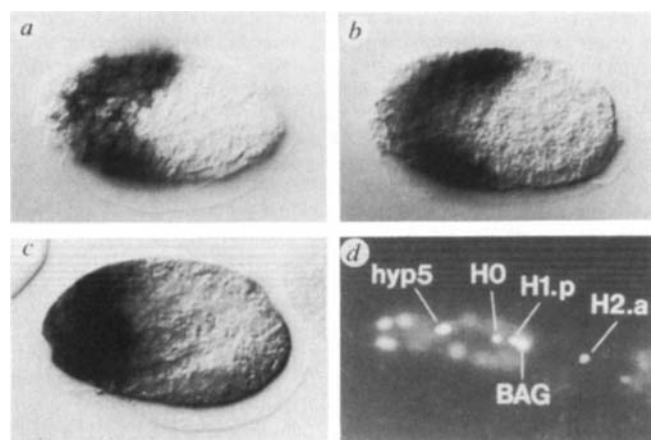
We cloned the *vab-3* gene by genetically mapping it with respect to genes placed on the physical map of the *C. elegans* genome and then by identifying DNA clones that rescued the *Vab-3* mutant phenotype in transgenic animals (Fig. 2a). Rescuing activity was located to a 22-kilobase (kb) *SalI-NheI* fragment and complementary DNA clones were isolated using parts of this 22-kb fragment as probes. We determined the sequence of one cDNA clone (B6) and found that it contained an open reading frame of 455 amino-acid residues; from analysis of B6

and other cDNA clones we believe that B6 encodes the complete VAB-3 protein. The predicted VAB-3 protein is most similar to the Pax-6 class of paired-domain proteins (Figs 2b, 3). PAX-6 proteins contain both a paired domain and a paired-type homeodomain⁹. The VAB-3 paired domain is 79% identical to that of the human PAX6 protein (102 of 128 residues), and the VAB-3 homeodomain is 93% identical to that of human PAX6 (56 of 60 residues) (Fig. 3). Other members of the Pax-6 family have extended similarity in the six residues LQLKRRK amino-terminal to the homeodomain, and in the seven residues KLRNQRR carboxy-terminal to the homeodomain; these sequences are partly conserved in VAB-3 (Fig. 2b). Complementary DNAs encoding proteins with the same homeodomain and C terminus as *vab-3* (encoded by exons 10–14), but with alternative N-terminal regions lacking the paired domain, correspond to the gene *mab-18* (ref. 5); these alternative N-terminal regions in *mab-18* are encoded by two exons (8 and 9) that are not present in *vab-3* cDNAs, and that are located between the *vab-3* exons labelled 7 and 10 in Fig. 2a.

To discover the molecular identities of *vab-3* mutations, we determined the sequences of the paired domain-encoding exons

FIG. 4 *vab-3* is expressed widely in head epidermal cells and neurons. a, b, Dorsal views of embryos at about 5 h embryogenesis, just before morphogenesis; dorsal and medial focal planes. Expression is seen in a set of 20–30 anterior dorsal cells, corresponding to the hypodermal cells of the head, and lateral sets of about 10 cells, of which some are probably future head neurons. c, 1.5-fold stage, left lateral view. *vab-3* transcripts are expressed in hypodermal and neuronal cells in a broad region of the head. d, Expression of the *vab-3*-GFP reporter construct (see below) in head cells of an L1 stage larva, left lateral view. This construct was expressed in the nuclei of most hypodermal nuclei of the head and in many neuronal nuclei; only those nuclei in the left lateral focal plane are labelled. We identified the hypodermal nuclei expressing *vab-3*-GFP in late-stage embryos and newly hatched larvae as the nuclei of hypodermal syncytia hyp3, hyp4, hyp5 and hyp6, the four most anterior nuclei of hyp7, and the lateral hypodermal cell H0. The lateral hypodermal cells H1 and H2 did not express *vab-3*-GFP, but the non-seam hypodermal descendants of these cells expressed *vab-3*-GFP. The BAG neurons²⁹ expressed *vab-3*-GFP strongly; we have not identified all weakly expressing cells.

METHODS. a–c, Whole-mount *in situ* hybridization of worm embryos was performed as described³⁰, using digoxigenin-labelled single-stranded DNA probes specific to the paired domain of *vab-3*. d, A *vab-3*-GFP reporter construct was made by cloning the 1.8-kb *SalI-BamHI* fragment of C56D7 into the vector TU#61 (ref. 15). We established transgenic lines in a *lin-15*(n765ts) background using rescue of the



lin-15 phenotype conferred by the plasmid pLin-15EK (ref. 31) as a marker. We made a strain carrying a chromosomally integrated array by γ -radiation mutagenesis using a ⁶⁰Co source. For photography, worms were anaesthetized using 1-phenox-2-propanol; fluorescence was visualized using the HQ-GFP filter set (Chroma Technology, Brattleboro, VT).

of nine *vab-3* mutants, and found that four carried alterations in the paired domain (Fig. 3). The allele *vab-3(e648)*, which causes a highly penetrant head morphogenetic defect and is therefore classified as a strong allele (A.D.C. and H.R.H., in preparation), encodes an opal stop codon instead of tryptophan at position 101, suggesting that *e648* causes a severe loss of *vab-3* function. Two phenotypically weaker alleles carry mis-sense changes in the paired domain: *sy66* encodes an asparagine instead of isoleucine at position 103, and *sy281* changes glycine at position 19 to arginine. The equivalent glycine residue is altered to a serine in the mouse *Pax-1* mutant *undulated*¹⁰. Based on the structure of the paired domain of the PRD protein¹¹, this residue probably contacts the minor groove of the paired-domain DNA-binding site. The strong allele *e41* carries an alteration in the conserved splice donor of exon 5 (AGC|gtgagt to AGC|atgagt) identical to that found in the aniridia patient NIKIT¹².

Vertebrate *Pax-6* genes are expressed in many parts of the developing brain, including the eye, the olfactory epithelium and the neural tube before neuronal differentiation^{9,13,14}, and the fly *eyeless* gene is expressed both in the embryonic nervous system and in eye imaginal disks⁴. To determine the cells in which *vab-3* might function in embryos, we analysed the expression pattern of *vab-3* transcripts using *in situ* hybridization. They were first detected in embryos at the 150-cell stage, in precursors of the head hypodermis and neurons and subsequently in the progeny of these cells (Fig. 4a, b). At the time when the neurons and hypodermal cells of the head begin to differentiate, *vab-3* transcripts were expressed in a broad domain across the developing head (Fig. 4c). We also analysed the expression of *vab-3* in larvae using the green fluorescent protein as a reporter¹⁵, and found that *vab-3* was expressed strongly in almost all anterior hypodermal nuclei and weakly in many head neurons, consistent with the defects in head morphogenesis and neuronal identity seen in *vab-3* mutants (Fig. 4d).

Despite the differences in the development of the head region in vertebrates, insects and nematodes, our results show that animals of all three phyla use the same type of gene to specify head-region cell fates. The high levels of *vab-3* expression in head hypodermal cells may parallel the expression of vertebrate *Pax-6* in surface ectoderm of the presumptive lens placode^{14,16}. The function of *Pax-6* genes in the specification of head ectoderm could thus be of comparable antiquity to the functions of the *Antp*-class homeobox clusters in specifying body regions¹⁷. It has been proposed that *Pax-6* genes might function as master control genes for eye development^{4,18,19}. Although *C. elegans* has been reported to have a weak response to light²⁰, it has no photoreceptor cells, unlike some marine nematode species. If ancestral nematodes lacked photoreceptors, our findings would suggest that the primordial role of the *Pax-6* gene family was in head-region specification and that it only later became involved in the development of eyes. □

Received 25 May; accepted 4 July 1995.

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ACKNOWLEDGEMENTS. We thank Y. Jin for help and advice concerning all aspects of molecular biology; Y. Zhang and S. W. Emmons for generously sharing unpublished data concerning *mab-18*; H. Chamberlin and P. Sternberg for *sy66* and *sy281*; P. Okkema and A. Fire for their embryonic cDNA library; G. Seydoux for advice concerning *in situ* hybridization; A. Na for maintaining worm strains; B. James for the DNA sequencing; and M. Basson, Y. Jin, M. Metzstein and S. van den Heuvel for comments about the manuscript. This work was supported by the US Public Health Service. A.D.C. was a Lucille P. Markey Visiting Fellow during part of this work, and his studies were supported in part by a grant from the Lucille P. Markey Charitable Trust. H.R.H. is an investigator of the Howard Hughes Medical Institute. The *vab-3* cDNA sequence has been deposited in the GenBank database, accession no. U31537.

Specification of sense-organ identity by a *Caenorhabditis elegans Pax-6* homologue

Yinhua Zhang & Scott W. Emmons

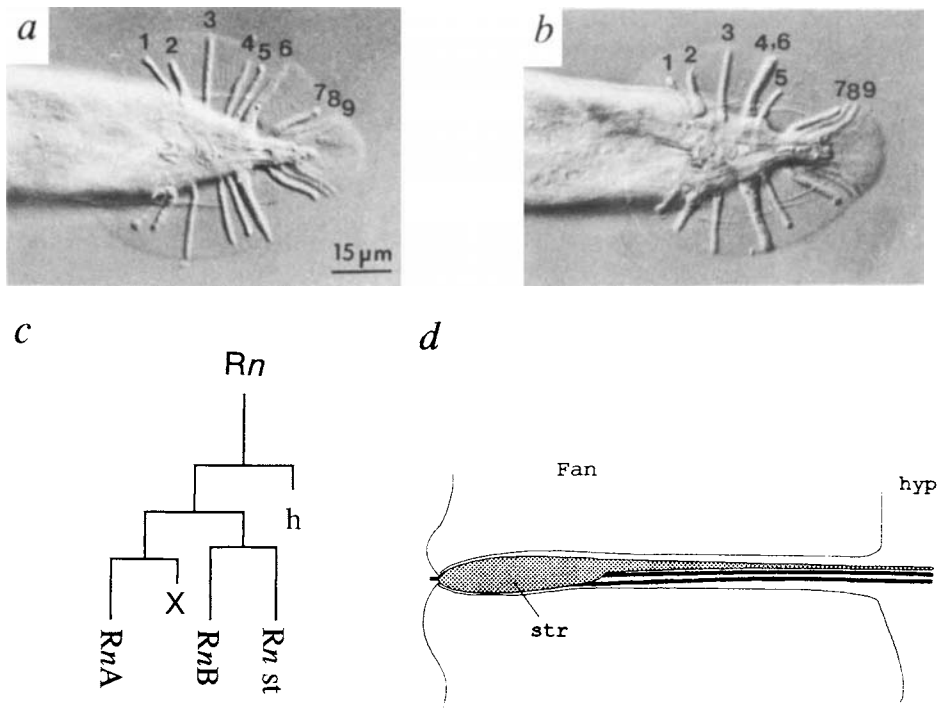
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The *Pax-6* transcription-factor gene, containing a paired domain and a paired-type homeodomain, is conserved in structure and ubiquitously present among Metazoa^{1–5}. It is required for development of the central nervous system, and is mutated in human aniridia, mouse and rat *small eye* and *Drosophila eyeless*⁶. We identified the *Pax-6* gene of the nematode *Caenorhabditis elegans* in genetic studies of male tail morphology^{7,8}. *C. elegans Pax-6* encodes at least two independent genetic functions. One, like other *Pax-6* genes, contains paired and homeodomains; this constitutes the genetic locus *vab-3* (ref. 9). The other, described here, is expressed from an internal promoter and contains only the homeodomain portion; this constitutes the genetic locus *mab-18* (ref. 7). The *mab-18* form of the gene is expressed in a peripheral sense organ and is necessary for specification of sense-organ identity. Its function in this context could be to regulate the expression of cell recognition and adhesion proteins required for sense-organ assembly.

Two *mab-18* mutations⁷, both recessive and fully penetrant, transform one pair of a set of nine pairs of male-specific genital sensilla (simple sense organs) known as rays (Fig. 1). We isolated the *mab-18* gene by complementation rescue of the ray defect with microinjected cloned DNA (Fig. 2 legend). The *mab-18* locus encodes a family of proteins, each containing a homeodomain and one of several alternative amino-terminal exons (Fig. 2). The homeodomain is identical at 56 out of 60 positions to vertebrate *Pax-6* (ref. 2), and at 55 out of 60 positions to *Drosophila eyeless* (ref. 4). In addition, several other features of the gene are similar or identical to other *Pax-6* genes: the gene contains a serine-, threonine- and proline-rich carboxy-terminal region and a long (921 bases) 3' untranslated region; the amino-acid sequence flanking the homeodomain is similar to that in other *Pax-6* genes; and the positions of introns within the homeodomain are identical.

Other *Pax-6* genes encode paired domains as well as homeodomains. The *mab-18* homeodomain-containing exons are also found in longer transcripts that include a *Pax-6*-like paired domain present in genomic DNA 4.3 kilobases (kb) upstream of the homeodomain (unpublished results; ref. 9). Mutations in

FIG. 1 Mutations in *mab-18* result in a transformation of the morphogenetic identity of a sensory ray. **a**, Wild-type *C. elegans* male tail, ventral view, Nomarski photomicrograph. Nine pairs of sensory rays project outward from the body within an acellular fan¹³. Each ray has a distinct morphogenetic identity characterized by anteroposterior position and by opening on the ventral surface, on the dorsal surface, or at the margin of the fan. In addition, ray 6 has a distinctive conical shape. **b**, *mab-18(bx23)* mutant male tail, ventral view. In *mab-18*, the morphogenetic identity of ray 6 is transformed to that of ray 4. Ray 6 is absent and a large fused ray (4,6) containing cells of both ray 4 and ray 6 (Fig. 3) is present in the position of ray 4. The characteristics of the other rays are not affected. **c**, Ray sublineage. Rays are generated by nine pairs of ray precursor cells (Rn , $n=1-9$), each of which expresses the ray sublineage during the late L3 and early L4 larval stages¹³. The ray sublineage generates the three cells of each ray¹³: RnA and RnB are neurons of distinctive ultrastructure; $Rnst$ is a support cell; x , programmed cell death. $Rn.p$ is a hypodermal cell (h), which normally fuses with other $Rn.p$ cells for $n=1-5$, and with the surrounding hypodermal syncytium for $n=6-9$ (ref. 13). **d**, Structure of a ray^{13,17}. At the ray tip the dendritic endings of the two neurons are surrounded by the structural cell (str) and held in an opening to the environment. The bulk of the



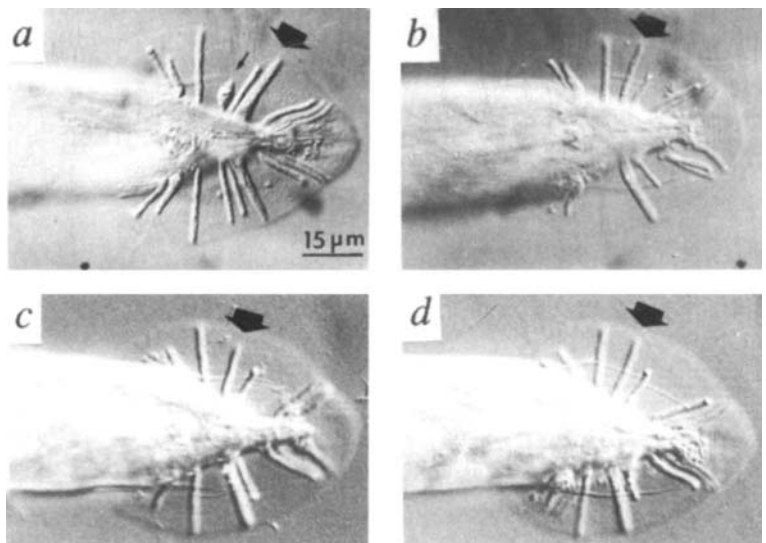
ray volume and all of its surface consists of hypodermal syncytium (hyp)¹⁷. Ray cell bodies are in ganglia lying anteriorly in the body.

the paired domain disrupt functions previously assigned to the genetic locus *vab-3* (ref. 9). *vab-3* mutations result in variable deformities of the larval and adult head, and of the adult male tail¹⁰, a phenotype different from the *mab-18* ray phenotype. We found that the *mab-18* mutations affect the same complex gene as the *vab-3* mutations. DNA sequence analysis showed that *mab-18(e1976)* consists of two missense mutations within the

homeodomain (Fig. 2c). This mutation has phenotypes in common with both *mab-18* and *vab-3* mutations and fails to complement both. Although *e1796* causes no head or tail deformity typical of *vab-3* mutations, it causes a gonad-cell-migration defect¹¹ identical to *vab-3(e648)* (A. Chisholm and H. R. Horvitz, personal communication; results not shown), and fails to complement *vab-3(e648)* for this defect (results not

FIG. 3 Cell-ablation experiments showing that in a *mab-18* mutant the specificities of the cell associations of ray 6 cells are transformed to those of ray 4. In **a** and **b**, the structural cell of ray 4 ($R4st$) and the neurons of ray 6 ($R6A$ and $R6B$) have been ablated in wild-type (**a**) and in *mab-18(bx23)* (**b**). The results show that in the wild type, $R6st$ forms ray 6 with normal tapering morphology in the absence of its associated neurons (large arrow in **a**), whereas in *mab-18* it forms a thin, cylindrical ray between rays 3 and 5 (large arrow in **b**). Therefore the structural cell determines the nature of the interactions between epidermal cells and ray cells that define ray position and morphology^{7,8}, and these characteristics for ray 6 are transformed by a mutation in *mab-18*. Ablations in **c** and **d** show that cells of both ray 6 (**c**, ablation of $R6$) and ray 4 (**d**, ablation of $R4$) are necessary to form a fused ray in *mab-18(bx23)* (compare the ablated sides (large arrows) with the unablated contralateral sides). The experiment reveals that in a *mab-18* mutant, ray 4 is unaffected (**c**), whereas ray 6 forms an uncharacteristic thin, cylindrical ray between rays 3 and 5 instead of a tapering ray at its normal position between rays 5 and 7 (**d**). When both ray 4 and ray 6 are present, they form a single, large ray at the position of ray 4, which contains cells from both lineages¹⁷.

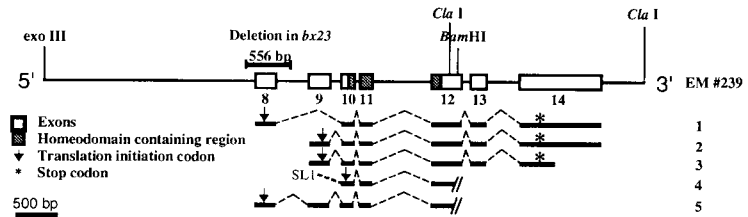
METHODS. Unilateral cell ablations were carried out with a laser microbeam⁸; in all figures, upper side is ablated, lower side is unablated. **a**, Ablation of $R4st$, $R6A$, $R6B$ in wild type, $n=2/2$; **b**, ablation of $R4st$, $R6A$, $R6B$ in *mab-18(bx23)*, $n=4/6$; **c**, ablation of $R6$ in *mab-18(bx23)*, $n=5/5$; **d**, ablation of $R4$ in *mab-18(bx23)*, $n=10/12$. The number of successful ablations is sometimes smaller than the total



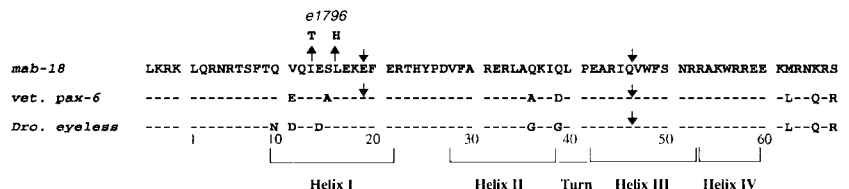
number of ablations because of inadvertent killing of other cells. In **a**, the ray-like lump indicated by the small arrow results from the tendency of neurons to extend into the fan in the absence of the ray structural cell. In **b**, ray 3 is absent from the unoperated side, which occurs at low frequency in wild-type animals.

FIG. 2 Structure of the *mab-18* locus and encoded gene products. *a*, Genomic region and structure of complementary DNAs. The genomic region shown (EM #239), as well as cDNAs 1, 2 and 3 when expressed from a heat-shock promoter, rescued the *mab-18* mutant-ray phenotype. The paired domain is 0.6 kb 5' of EM #239 (results not shown; ref. 9). Exons are numbered sequentially with exon 1 corresponding to the most 5' *vab-3* exon⁹. In cDNA 4 the spliced leader sequence SL1 (ref. 18) is joined to the splice acceptor of exon 10. The 5' ends of exons 8 and 9 are defined by the 5' ends of cDNAs 1 and 3, respectively. The 5' end of cDNA 2 is 19 bases downstream of the 5' end of cDNA 3. The structure of all cDNAs was determined by DNA sequencing, except for that of cDNA 5, which is proposed on the basis of cross-hybridization data (a potential in-frame splice acceptor sequence is present at position 31 within exon 9). *b*, *mab-18* nucleotide and deduced amino-acid sequence (single-letter code). The homeodomain is underlined. Polyadenylation signal sequences (T. Blumenthal, O. White and C. Field, personal communication) are boxed. cDNA 3 is polyadenylated 14 bases downstream of the first polyadenylation signal. The Ser-Thr-Pro rich region also found in other Pax-6 proteins is indicated by individually underlined amino acids. ↓, Positions of introns, c, Comparison of the *mab-18* homeodomain (numbered 1–60) with Pax-6 homeodomains of vertebrates² (known vertebrate Pax-6 homeodomains are identical) and *Drosophila*⁴. Sequence identity or similarity extends 4 amino acids on the amino-terminal side, and 7 amino acids on the carboxy-terminal side. *mab-18*(e1796) consists of two single-base changes (105ATT to ACT and 114CTT to CAT), resulting in the amino-acid sequence changes shown by ↑, ↓. Positions of introns.

METHODS. Genes flanking *mab-18* (X) were determined to be in the order *unc-115-mab-18-daf-12-egl-15* in a multifactor cross. A recombination metric derived from the known distance between *daf-12* and *egl-15* was used to place *mab-18* 255 ± 150 kb to the left of *daf-12*. Cosmids (A. Coulson) were coinjected with plasmid pRF4, carrying the dominant *rol-6* marker¹⁹. Overlapping cosmids C33D3, CB11, MB4 and F14F3 had rescuing activity. EM #239 was derived by exonuclease III deletion of MB4. cDNAs 1 and 2 were isolated from a λgt11 cDNA library²⁰, and cDNA 3 as well as four shorter forms consisting of more 3' regions (not shown) were isolated from a mixed-stage 1–2-kb cDNA library²¹. cDNAs 4 and 5 were isolated by amplification by polymerase chain reaction after reverse transcription of total RNA (RT-PCR) of mixed-stage animals (5'-RACE System, Gibco BRL) by means of a primer within exon 12. Two additional cDNAs isolated by RT-PCR contained paired domain sequences spliced to exon 10, and represent *vab-3* transcripts (results not shown; ref. 9). cDNAs 1, 2 and 3 were tested for rescue of the *mab-18* ray defect after cloning into heat-shock-expression vector pPD49.78 (ref. 22). Transgenic animals were subjected to heat shock (31 °C for 3 h or 33 °C for 20 min) during late L3 or early L4 larval

a*b*

exon 8	TCCTCACATCACTCCTTTCTATCTGATTGGGATGCTTCTGACACCCGACAGTCGGGCC	60
	CTACCGACCGTTTGGGGGAACAAGCTCGAAAACTGGGCTCCAACGCTGATTTATCTCGT	120
	GCTCCGTCGATCTACTGTATTCATCGTCTGCTTACGCGGAATGCAACGCACTGATTG	180
	TATACAATAAGCGACACCCCTTCAAGATGCGACCAATCGCTAATACGAG	229
	Y T I S D T L P R C D Q S L I R D	
exon 9	CATACAGTCTCTTTCAAGAGATGATATCAGCACACGCTCCCATAGTGTCTATCAAGGCA	59
	CGGTACCAACTCGCGAGCTTCCACTATTGTAAGACCGCTGTCGGTAAATTTCTCT	119
	CCGT	123
	P Y	
exon 10-14	ATGAGGACCAAAAACCTCCAACTGAGCCAGAAGTGACGCGCAGCCAGGATCGGATTG	59
	E D Q K P P T E P E D D A A A R M R L	
	AAACGGAAGTTGCAAGAAACAGAACCTCGTTTACGCAAGTCCAGATTGAGAGTCTTGAA	119
	K R K L Q R N R T S F T Q V I E S L E	
	AAAGAAATTTGAAAGGACTTATTCAGATCTTTTTCGAAAGGAGCGCTTTCGCGCAGAAA	179
	K E F E R T H Y P D V F A R E R L A Q K	
	ATTCAACTACCAAGCAAGCAAGTATGCTTCTCGAATCGCGCGCAAAATGGAGG	239
	I Q L P E A R I Q V W F S N R R A K W R	
	AGAGAGAAAAGATGAGAAACAAGCATCATCGCGCACCATTGATTCATTCATTCAT	299
	R E E K M R N K R S S G T M D S S L S N	
	GGAACGCCAACGCCAACCGCGGATCAGTGACTGGAAGTAACATGACAAATCCGATTGGA	359
	G T E T E T E G S V T G S N M T N P I G	
	TCTCCGCCAGCAGCGCAATCGATTCCCTTCCAACTTCGCGCAATCTACCGCAACA	419
	S E A S T P N R F E S N N S A N L E T T	
	AACTTGTGCTCAACAGAGTCAAGTATGCTGGCTCAGTCAGCCAGCAATGGATCCA	479
	N F V E Q T S Q M Y A G L S Q E A M D E	
	TATTCATTGGAATGCAATGGTTTTCATGCTTCCATATCCACAAGTAACAGATTTC	539
	Y S F G I A N G F S M A E Y E Q V T D F	
	CAACCATCATATGTTTCAAGAGATCGCGGTATGATTTCCATATCCAAAGATCGCG	599
	Q E H H M F Q G R S E Y D F E Y R M E	
	ACCAATGGGATGGTTTCCAACTCAATGAGTCCCGCAACACCGCGTAGGAGACATT	659
	T N G H G F Q Q S M S E A T A T A V G D I	
	CGGACACTTCTTCTGGAATGAGCTACCGGTTTCTGAGTGTCTCAATTCATTCAGCCG	719
	E T L S S G M E L E V S A V L N S I D E	
	TCTCTGACTCACTACAAATGCAATGAGTCAAGTCAACAGCAATATTGAGAGA	779
	S L T H S Q M H E L S D L T Q E H Y W R	
	CCTCAGTAGAGATAGTTCTAAAGCAGATTGAATATATATATCCCTATTAAATTTAAGA	839
	E Q *	
	CATTAAATTCAGTTTGTCTACCATTTTTTTTGTTCATTCCTTTAATTCAGTTCAGTCCA	899
	CATCATCATCTCGTCTCTTTCTTTTGGTTCAATTTCTTTTCTGCTGCTATAAATCTTCTGG	959
	TTTCATGAAATTTCTCAAAATCAATTAAGATCAAGTCTAATCATGGTTTCATTTTGCA	1019
	CTTTTTCGAATGTTAACTCAATTAATTTTAAATAGAAATCAATGCAAACTTTCTATGAGCT	1079
	TTTATATATTTTCAACCCCTGGTCCCTTTTCTTCAATTCATGAGTGTCAATATATCA	1139
	ATCCCAATCTTTATGCTAGATTTTGTAGACAGTACATTTTGTGTCTCCATTTAAATG	1199
	GAACATGTAGATATGCTCTACAGAAATCGTGCACAAATGACATTTGCCCCACGTCC	1259
	CCCCCCCCCTCTCTTAAATTTCAAACTTCTTCCAGAGTTTTCATATGTATCATCAT	1319
	TTTTTATCTGTTGAGCATGAAATGTGTTTTCGTTTTCAACTATATATTTCTAGGTTT	1379
	AACATGATTCATTTGATTTATGACATATTTGAAACGAAATCTGTGTATCATTTAA	1439
	CTCAGTATTTGTCAGTATTTTCTCAATTTTAAACCTTAGAAATCTACCACTTTCCATG	1499
	TAGATGTGCAATTAATTTTATCTTGGAGTGAAGCAATTTTGGACATTTATAGTGAATAA	1559
	GAAAACGTTTCAATTTGTTTCATATCATGCAATTCATCTGTTGTGTTTCTTTTTCGAAA	1619
	TTTGGACATTTCTTCTCTTTTGAACCGTCTCTCATGTTATATTTGTCGAAAAGTA	1679
	AAATACCTAATGTTTATAATTTGTTGTTACTTTAAAAAATAAAAAA	1725

c

stages. The frequency of rescue of transgenic animals with unintegrated arrays was about 1% and was similar for all three cDNAs; frequency of rescue after integration of cDNA 2 was 9% ($n=864$ sides). GenBank accession numbers for *mab-18* cDNAs 1 and 2 are U29145 and U29184, respectively.

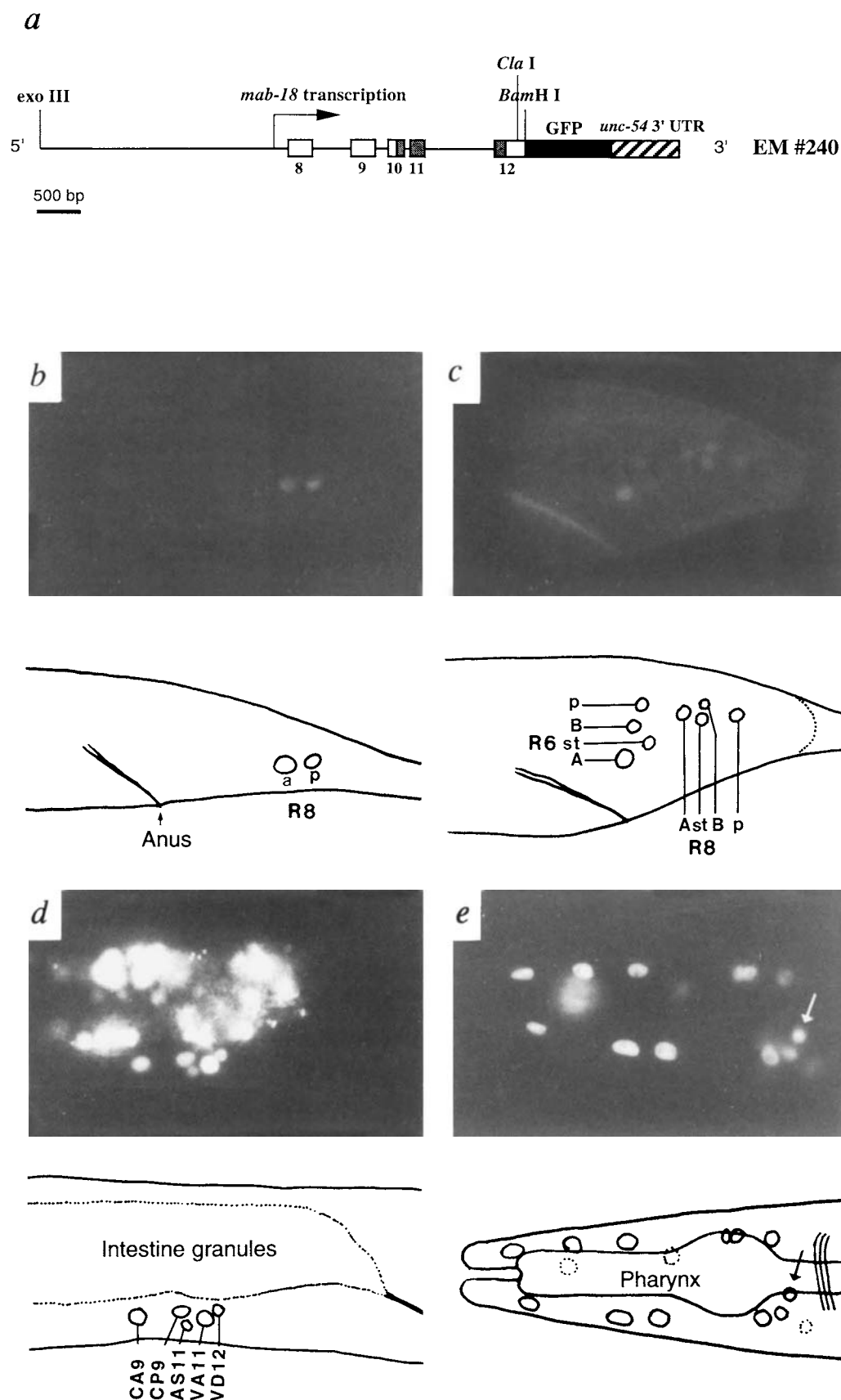


FIG. 4 Expression pattern of a *mab-18* reporter gene. **a**, The reporter consisted of the minimal *mab-18* rescuing region (EM #239, Fig. 2a) with the C terminus and 3' untranslated region (UTR) replaced with the GFP (green fluorescent protein) gene containing the *unc-54* 3' UTR (TU #61²³). The reporter GFP is localized to the nucleus, presumably targeted by a nuclear localization signal present on the *mab-18* sequence. **b**, Expression in R8.a and R8.p in late L3. **c**, Expression in ray 6 and ray 8 cells in late L4. Expression in ray 6 cells remains in adults, and disappears in ray 8 cells. **d**, Expression in neurons descended from P11.a in the preanal ganglion¹³, L4 male. **e**, Expression in the head hypodermis (hyp 1 6 and a few anterior hyp 7 nuclei) and a pair of bilaterally symmetrical neurons in the head anterior of the nerve ring (arrow), L4 male.

METHODS. The *mab-18*-GFP reporter (EM #240) (40 ng ml⁻¹) was introduced into *lin-15*(e765ts);*him-5*(e1490) hermaphrodites by microinjection with pL15EK (20 ng ml⁻¹), containing a copy of *lin-15*(+) as a selectable marker²⁴. Integrated lines were isolated after γ -ray irradiation. The green fluorescence was observed with a Carl Zeiss FITC filter set (450–490 nm excitation filter, FT510 barrier filter, 515–565 nm emission filter) with a Zeiss Axio-plan microscope.

shown). *mab-18*(*bx23*) is a 556-base-pair (bp) deletion covering exon 8 and additional flanking sequence (Fig. 2a). Exon 8 is absent from paired-domain-containing transcripts isolated so far (ref. 9; unpublished results). *mab-18*(*bx23*) and *vab-3*(*e648*) are mutually complementing and genetically closely linked (<0.02

map units), consistent with the conclusion that they disrupt independent functions of the same gene (results not shown). Thus the *C. elegans* *Pax-6* gene encodes at least two separable functions, one encoded by transcripts containing a paired domain and a homeodomain, required for gross morphology of

the head and tail, and one encoded by transcripts containing a homeodomain only, required for development of a ray. A form of the *Pax-6*-encoded protein containing only the homeodomain has previously been reported from quail retina¹².

To learn more about the function of the *Pax-6* gene disrupted in *mab-18* mutants, we investigated the cellular basis of the ray defect in *mab-18* (*bx23*) by selective ablations of ray cells. During ray development, several heterotypic and homotypic cell associations are formed^{7,13}. In *mab-18* mutants, the specificity of the cell associations of the structural cell and neurons of ray 6 have been transformed to those characteristic of ray 4 in the wild type (Fig. 3). In addition, the hypodermal cell generated by the ray 6 sublineage, R6.p, fuses with R1.p–R5.p, as does R4.p in the wild type, instead of with the hypodermal syncytium (results not shown). Thus it appears that in *mab-18* mutants the identity^{7,8} of the entire ray 6 sublineage has undergone an anterior transformation and resembles that of ray 4.

Reporter gene studies revealed the presence of a promoter in the intron separating the paired and homeodomains (Fig. 4). This promoter was activated in the ray 6 sublineage during the L4 larval stage and remained active in adult males. Therefore the putative MAB-18 transcription factor is most probably expressed and acts within ray 6 to determine its identity. One possibility is that it acts directly to regulate the expression of the cell recognition and adhesion genes postulated to characterize this ray and guide its independent assembly. The internal promoter was also activated in a number of other cells (Fig. 4), consistent with the many effects of mutations at this locus.

The results reported here and by Chisholm and Horvitz⁹ show that the *Pax-6* gene of *C. elegans* acts during development in several body regions. Its function is likely to be a general one in defining cell fate. Defects in cellular associations and assembly were previously proposed to account for the head defects found in *vab-3* mutants¹⁰. Thus one function of the *C. elegans Pax-6* gene appears to be to regulate the cell recognition and adhesion

functions expressed by neurons and support cells. In addition, *vab-3* mutations result in specific transformations in the fates of epidermal blast cells in the head^{9,14} and tail¹⁵. Thus the *C. elegans Pax-6* gene acts both in aspects of terminal cell differentiation and in specifying the properties of blast cells. Additional affected cells yet to be defined may include those responsible for the reported *C. elegans* photo-response¹⁶. A role of *Pax-6* in the photoreceptor cell of a primitive ancestor could account for the conserved function of *Pax-6* in the divergent eyes of arthropods and vertebrates. □

Received 30 May; accepted 24 July 1995.

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ACKNOWLEDGEMENTS. We thank A. D. Chisholm and H. R. Horvitz for communicating their results prior to publication. N. Baker and D. Stein for comments on the manuscript, and members of the laboratory for discussion. Some nematode strains were received from the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. This work was supported by the NIH.

Neural computation of log likelihood in control of saccadic eye movements

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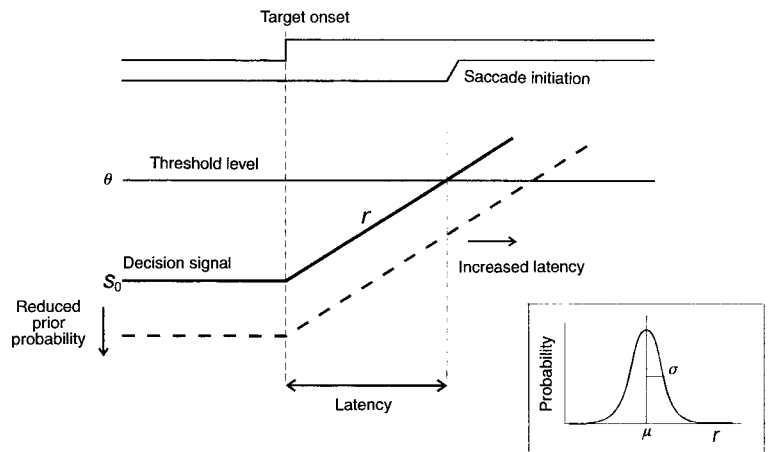
THE latency between the appearance of a visual target and the start of the saccadic eye movement made to look at it varies from trial to trial to an extent that is inexplicable in terms of ordinary 'physiological' processes such as synaptic delays and conduction velocities. An alternative interpretation is that it represents the time needed to decide whether a target is in fact present: decision processes are necessarily stochastic, because they depend on extracting information from noisy sensory signals¹. In one such model², the presence of a target causes a signal in a decision unit to rise linearly at a rate r from its initial value s_0 until it reaches a fixed threshold θ , when a saccade is initiated. One can regard this decision signal as a neural estimate of the log likelihood of the hypothesis that the target is present, the threshold being the significance criterion or likelihood level at which the target is presumed to be present. Experiments manipulating the prior probability of the target's appearing confirm this notion: the latency distribution then changes in the way expected if s_0 simply reflects the prior log likelihood of the stimulus.

An appropriate framework for considering decision processes based on uncertain evidence is provided by likelihood theory^{3,4}. A prior estimate of the likelihood L of some hypothesis H_1 (in this case, that there is in fact a target) relative to some reference hypothesis H_2 is modified on the basis of an observation E to form an updated or posterior likelihood L' . The effect of the observation is then to increase the logarithm of the perceived likelihood by a fixed amount S representing the support for H_1 against H_2 provided by E :

$$\log(L') = \log(L) + S, \quad \text{where} \quad S = \log \frac{\text{prob}(E|H_1)}{\text{prob}(E|H_2)}$$

In a case where there is a steady flow of information about the existence of the target, providing support at a certain fixed rate, log likelihood would therefore be expected to rise linearly, and a decision to take action would most naturally occur when this decision signal reaches some criterion level (broadly equivalent, for instance, to 'significance levels' in common statistical tests). The correspondence of such a decision system with the model (Fig. 1) proposed as the basis of saccadic latency is obvious: the decision units for different hypotheses can simply race against one another, the hypothesis enjoying greatest support reaching threshold soonest and therefore winning a saccade. A similar system, embodying a race between saccadic trigger units, was suggested 20 years ago in a different context by Robinson⁵. Where the model in Fig. 1 differs from others^{6,8} that also invoke a rise to threshold is that the rate of rise varies from trial to trial, but not during a trial: it is therefore not a random walk or diffusion model, and does not embody stochastic variation of the criterion level. It is tempting to identify this rising decision

FIG. 1 Model of the proposed decision process for initiating saccades. A decision signal, initially s_0 , starts to rise in response to the target at a constant rate r until it crosses a fixed threshold level θ : a saccade is then initiated. The rate r varies randomly from trial to trial, with a gaussian distribution (inset) of mean μ and variance σ^2 ; s_0 depends on the prior probability of the target being present, and when reduced causes an increase in latency.



signal with the similar rise of activity found before saccades in posterior parietal cortex and frontal eye field neurons⁹⁻¹¹.

We tested the applicability of such a description by systematically varying the prior probability of the appearance of different targets. Subjects' horizontal eye movements were measured in trials in which they were presented first with a central fixation light, and then, after a random delay in the range 0.5–1.5 seconds, with the sudden appearance of a second light randomly to left or right (the fixation light remaining on), to which they made a saccade. In different experiments the proportion of occasions on which the target went to left or right was systematically altered, to generate different prior probabilities for appearance on each side (50:50, 75:25, 90:10, 95:5). After some practice with a particular setting, subjects' latencies gradually changed to reflect the new prior probabilities, the latencies to

expected targets becoming shorter, and to unexpected ones longer. Once these changes seemed to be complete, which took many hours of practice, the experimental data were gathered.

Latencies were measured in 10-ms bins and used to construct cumulative plots on a probit scale with a reciprocal time axis as shown in Fig. 2. If the variation of r is gaussian with mean μ and variance σ^2 , the latency will be $(\theta - s_0)/r$ on any one trial, and its distribution will fall on a straight line with median $(\theta - s_0)/\mu$, intercepting the $t = \infty$ axis at $m = \mu/(\sigma\sqrt{2})$, this value being independent of s_0 and θ . Although the great majority (90–95%) of saccades have latencies that fall on such a distribution, there are circumstances in which one finds more saccades with very short latencies than would be expected. With data sets large enough to establish the form of this secondary distribution, these faster saccades usually lie on a second, shallower, straight line

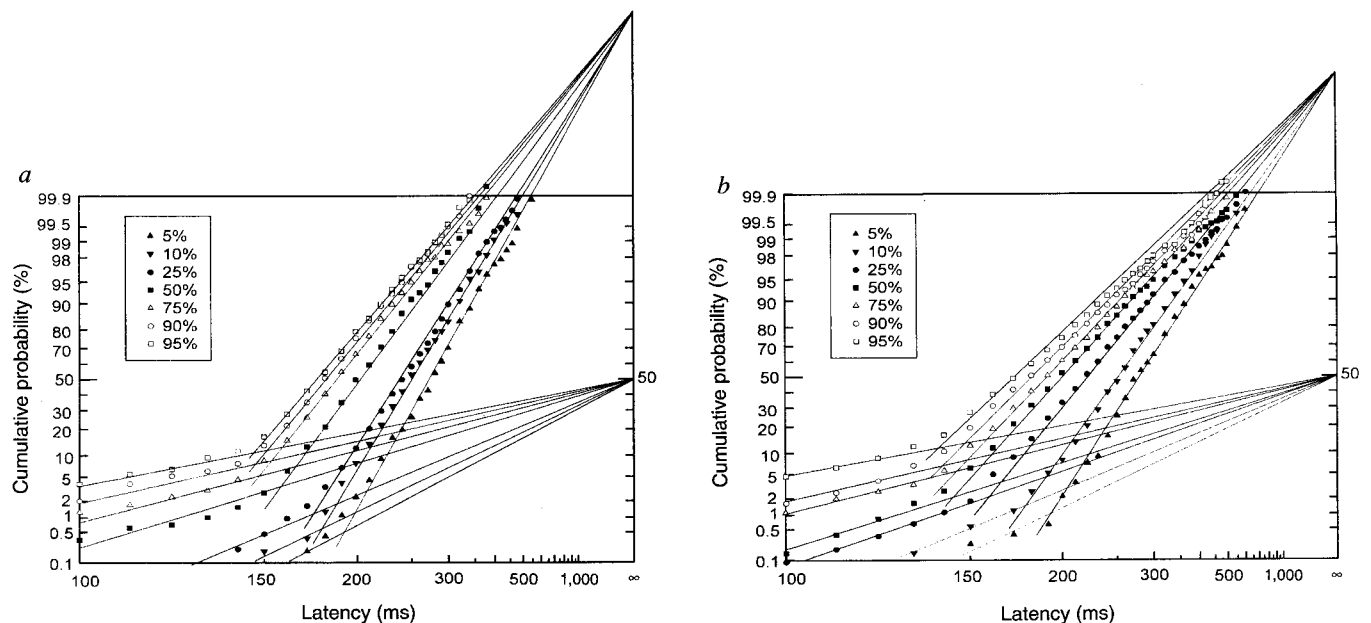


FIG. 2 Saccadic latency distributions measured in two subjects (a, b) and plotted in the form of cumulative percentage probability, on a probit scale, as a function of reciprocal latency. Latencies were measured by a computer-based system¹⁹ using an infrared oculometer, with a bandwidth of more than 500 Hz, in 10-ms bins. At the start of a trial a central fixation light-emitting diode (LED) was illuminated, followed after a random interval in the range 0.5–1.5 s by the illumination of a second LED 15° randomly to right or left. The percentage of trials in which the target appeared to right or left could be varied, leading to changes in the subject's perception of the probabilities of appearance of each target. Each of the curves corresponds to a different such prior-

percentage probability, as shown in the inset. Each distribution shows a main, linear portion, and the more probable targets show in addition an express component that is also linear. In each case the effect of altering target probability is to alter the slopes of the components, but in such a way that the intercept with the right-hand axis (infinite latency) is constant. This corresponds to alteration of the threshold criterion level for the decision signal in the proposed model. The lines corresponding to the main parts of the distributions are drawn so as to minimize χ^2 for the relevant data points, with the constraint that all had to pass through the same intercept (for infinite time), chosen to minimize χ^2 overall; the express guidelines are drawn by eye.

TABLE 1 Goodness of fit

Probability	Subject M.W. (Intercept = 6.20)				Subject V.R. (Intercept = 4.60)			
	Trials	Median	d.f.	χ^2	Trials	Median	d.f.	χ^2
0.05	567	273	16	25.64	530	283	18	27.89
0.1	511	250	15	13.4	735	256	20	27.46
0.25	520	241	15	9.89	869	218	20	20.51
0.5	1,366	202	16	20.48	1,553	196	19	21.15
0.75	1,535	187	14	21.66	2,662	188	18	23.37
0.9	4,569	179	14	23.68	6,565	179	17	25.06
0.95	10,969	176	14	46.44*	9,624	171	19	51.32*

* $P < 0.05$.

crossing the main distribution, normally intercepting the $t = \infty$ axis at 50% probability ($m = 0$). They become prominent with advance cueing of the target, and probably correspond with 'express' saccades^{12,13}, although the classification of fast saccades is somewhat controversial¹⁴, partly owing to the use of raw rather than cumulative histograms, which may present a misleading appearance. The 'express' distribution can be accounted for by supposing a second decision unit, with $\mu = 0$ and a large σ , in parallel with the first and perhaps identifiable with the parallel pathways involving visual cortex and colliculus rather than frontal eye fields, that have been proposed to explain the existence of express saccades^{15,16}. However, although these faster responses feature in the data presented here, it is the main part of the distribution with which we are primarily concerned. If the target has appeared so often that the subject's estimate of its prior likelihood is high, then the starting level s_0 should be closer to the threshold θ , and the latency should be reduced. In fact the prediction is more stringent than that: because the change is in s_0 rather than in μ or σ , the distribution of latency should alter in a very specific way: the slope of the plot should become shallower, but the intercept with the $t = \infty$ axis should remain unchanged.

As can be seen in Fig. 2, the subjects show very similar changes in their distributions as the prior probabilities are altered. Under all conditions the slowest 90–95% of saccades fall satisfactorily on the straight lines predicted by the model. (In cumulative plots such as these, the weight of data points in the middle of the distribution is necessarily far greater than further out, where a single point may depend on only one or two observations out of several thousand. Here, a χ^2 test was used to determine whether the observed distribution fitted the expected relation-

ship, using only the main part of the distribution and ignoring the express component: because the predicted form of the entire distribution is non-analytical it cannot easily be used in such tests. For each subject, the value of the intercept m yielding the smallest total value of χ^2 was found, and the line of best χ^2 fit for each of the seven conditions through that intercept was determined (Table 1). For both subjects, only the lines for $P = 0.95$ fail the χ^2 test at the 5% level. One reason for this may be that the data sets for $P = 0.95$ were extremely large (around 10,000 individual trials each) and were necessarily obtained over the course of many days; because latency appears to be subject to long-term variability having some of the characteristics of $1/f$ noise, this means that the overall variability does not decrease as much with increasing number of trials as is assumed in the χ^2 test.)

When the target is relatively unexpected, the median latency is long and there is little sign of the express component. As prior probability increases, the main distribution becomes faster and shallower, with an increasing number of express responses. In each case, the prediction that the infinite-time intercept is constant is evidently obeyed, implying that the reduction in latency caused by increasing prior likelihood is indeed through a reduction in the distance $(\theta - s_0)$ that the decision signal has to rise, rather than a change in its mean rate of rise μ (which would have displaced the curves parallel to one another). In terms of the model it is natural to interpret this reduction of $(\theta - s_0)$ as being due to an increase in s_0 rather than a decrease in the threshold, although our data cannot distinguish between these two cases.

A further, more stringent and quantitative, test can be made: if the decision signal is indeed a representation of log likelihood, then s_0 should represent the prior log likelihood L ; consequently median latency, directly proportional to $(\theta - s_0)$, should be reduced linearly in proportion to L . Figure 3 plots median latency of the main distribution against log prior probability, showing that median latency, and thus s_0 , does indeed appear to vary linearly with log likelihood. The slope of this relation is equivalent for both subjects to some 76 ms per log unit of support, which could be taken to be the rate at which information is being used to reach a decision. One might wonder why the lines extrapolate to some 175 ms latency at 100% prior probability, rather than to something nearer zero. The reason is that 'prior probability' here means the probability of the target occurring eventually, but the conditions of each trial were deliberately arranged (by randomizing the wait period after the first cue) so that the subject could not know exactly when the target would appear: subjects can respond with zero latency to targets whose time of appearance and position are both certain^{17,18}. In the 50:50 case, for instance, only 40 of the 200 ms total latency can be ascribed to uncertainty regarding the position of the target. It would be interesting to extend these observations to include cases where the prior time-uncertainty is increased or reduced, when we would predict similar changes in the shapes of the distributions and in median latency, also as a consequence of giving subjects instructions likely to lead them to alter θ , the criterion level for accepting that a target is actually present. $\square$

Received 4 August 1994; accepted 21 July 1995.

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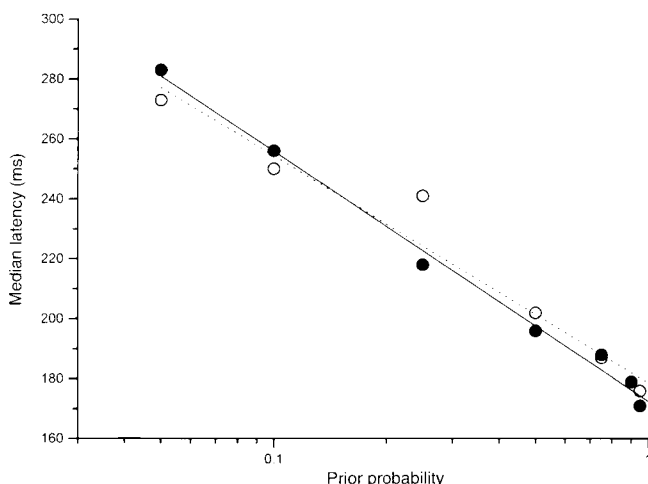


FIG. 3 Median target latency plotted as a function of log prior probability of a target being present, for the two subjects; the straight lines are apparent linear least-square fits (dotted line, open circles, M.W., $r = -0.981$, slope = -76 ms per log unit; solid line, filled circles, V.R., $r = -0.997$, slope = -83 ms per log unit), differing significantly in neither slope nor offset.

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ACKNOWLEDGEMENT. We thank V. Ratcliffe for spending many hours as an additional subject, and J. D. Schall for valuable criticism and discussion.

Real-time measurement of transmitter release from single synaptic vesicles

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NEUROTRANSMITTER release is mediated by Ca^{2+} dependent exocytosis of synaptic vesicles¹. Neither the amount of transmitter released from individual synaptic vesicles nor the kinetics of this process have yet been directly determined. Using carbon fibres as electrochemical detectors^{2,3}, we have measured release of the neurotransmitter serotonin from cultured neurons of the leech⁴. This technique allowed us to monitor transmitter discharge from single synaptic vesicles as spike-like oxidation currents at high time resolution, providing new insight into the mechanism of neuronal exocytosis. Two types of signals were characterized, corresponding to exocytosis of small clear and large dense core vesicles present in these cells. A small vesicle discharges about 4,700 transmitter molecules with a time constant in the region of 260 μs , whereas large vesicles release their content of approximately 80,000 molecules with a time constant of about 1.3 ms. Release from both vesicle types is initiated rapidly, with a rise time of less than 60 μs , suggesting an abrupt opening of a preassembled fusion pore.

Isolated Retzius cells of the leech *Hirudo medicinalis* were used to study transmitter release under amperometric recording conditions. Single Retzius cells synthesize, store and release serotonin as their only classical transmitter and form a rapidly acting serotonergic synapse if co-cultured with a postsynaptic neuron^{4,5}. We manipulated the tip of a carbon fibre to the distal end of the axonal stump of a single Retzius cell, gently touching its membrane. In the presence of 5 mM extracellular calcium, an action potential evoked a complex current transient with spike-like current deflections (Fig. 1a). Lowering the extracellular calcium to 1 mM reduced the amplitude of the overall response and allowed us to resolve single, well separated spikes which follow the action potential with a short delay. These signals were greatly decreased when the potential applied to the carbon fibre (+650 mV) was reduced to +300 mV and disappeared upon further reduction, as expected for serotonin oxidation. When the detector was moved away from the cell, the signals became smaller and slower. Furthermore, signals were reversibly blocked by superfusion with 100 μM Cd^{2+} , demonstrating their dependence on calcium influx during the action potential (data not shown). These results suggest that the spike-like current transients represent single exocytotic events, releasing quantal packages of oxidizable serotonin, whereas signals in high Ca^{2+} reflect the summation of many events as well as transmitter release from more distant locations.

Small events were regularly seen within the first few milliseconds after an action potential (Fig. 1b). In addition, we occasionally observed large current transients following the action potential with a longer delay. These events were clearly distinguishable from small events with respect to charge, amplitude and kinetic parameters (Table 1). This suggests that small and

TABLE 1 Properties of small and large events evoked by single action potentials in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}

	Small events		Large events	
Charge (fC)	3.0 $\pm$ 0.1	(2.8)	47.4 $\pm$ 3.0	(37)
Amplitude (pA)	5.5 $\pm$ 0.1	(4.7)	19.5 $\pm$ 1.5	(12)
50–90% risetime (μs)	90 $\pm$ 5	(50)	475 $\pm$ 23	(320)
Half-width (μs)	595 $\pm$ 40	(420)	3,660 $\pm$ 230	(2,540)
Latency (ms)	4.5 $\pm$ 0.5	(1.6)	16.7 $\pm$ 1.7	(7.5)
Frequency per AP	0.83 $\pm$ 0.2		0.05 $\pm$ 0.03	

Small ($n = 220$) and large ($n = 140$) events were defined as current transients with a charge smaller or larger than 8 femtocoulombs, respectively (Fig. 2b). Each event was characterized with respect to area (charge in femtocoulomb), amplitude (peak value in picoampere) and kinetic parameters. A 50–90% rise-time reflects the time between the 50 and 90% value of the peak amplitude. The 50–90% rise-time was chosen to avoid including 'foot' signals which often precede large events (Fig. 3b). Half-width is defined as width of the current transient at 50% of its peak amplitude. Latency is given as time between the peak of the action potential (AP) and the onset of the current transient. 8 cells (380 APs) and 34 cells (3,600 APs) were analysed to obtain frequency data (during the first 50 ms after the AP) for small and large events, respectively. Measurements are given as mean $\pm$ s.e.m., and numbers in brackets represent median values of the corresponding frequency distribution. Similar values with respect to charge, amplitude, rise-time and half-width were obtained for both types of events recorded under spontaneous conditions (data not shown).

large events reflect exocytosis of distinct classes of synaptic vesicles. Electron microscopy confirmed that Retzius cells contain distinct populations of small clear vesicles (SSV) and large dense-core vesicles (LDCV) at the recording site (Fig. 2d). SSV are organized in clusters directly adjacent to the plasma membrane, whereas LDCV are mainly found at more remote locations. The differential spatial distribution of SSV and LDCV fitted with the different time-dependent coupling of small and large events to single action potentials (Fig. 1c). Small events occur most frequently within the first 2 ms after the peak of an action potential and then sharply decline in frequency. In contrast, large events were never observed within the first millisecond after the action potential but show an increased release probability over the next 10 ms, which then slowly declines. We conclude that small events are due to exocytosis of SSV, whereas large events reflect transmitter release from LDCV.

The frequency of large events was increased 4-fold by stimulating cells with trains of action potentials (Fig. 1e), consistent with observations in other systems showing that secretion of neuropeptides (stored in LDCV) is greater with trains of action potentials than with single stimuli⁶. Large events occurred more often at the end of the train and their frequency remained constant over many successive stimuli. Small events were seen more frequently after the second and third action potential of a single train but their overall frequency decreased during subsequent trains of stimuli. The decrease in small events is comparable to the attenuation of postsynaptic signals observed during high-frequency stimulation of Retzius cells⁷ suggesting that, at the synaptic site, transmitter release is mediated predominantly by SSV.

To confirm our hypothesis that both classes of signals reflect vesicle-mediated exocytosis, cells were microinjected with tetanus-toxin light chain (TeTx-Lc) which blocks transmitter release by cleaving the synaptic vesicle protein synaptobrevin^{8,9}. Both signals were completely abolished under all stimulus conditions tested (Fig. 1f).

Small and large events showing similar properties also occurred spontaneously (Fig. 2a). The charge distribution, derived from time integrals of the current transients shows two clearly separated peaks consistent with transmitter release from the two vesicle populations, SSV and LDCV (Fig. 2b).

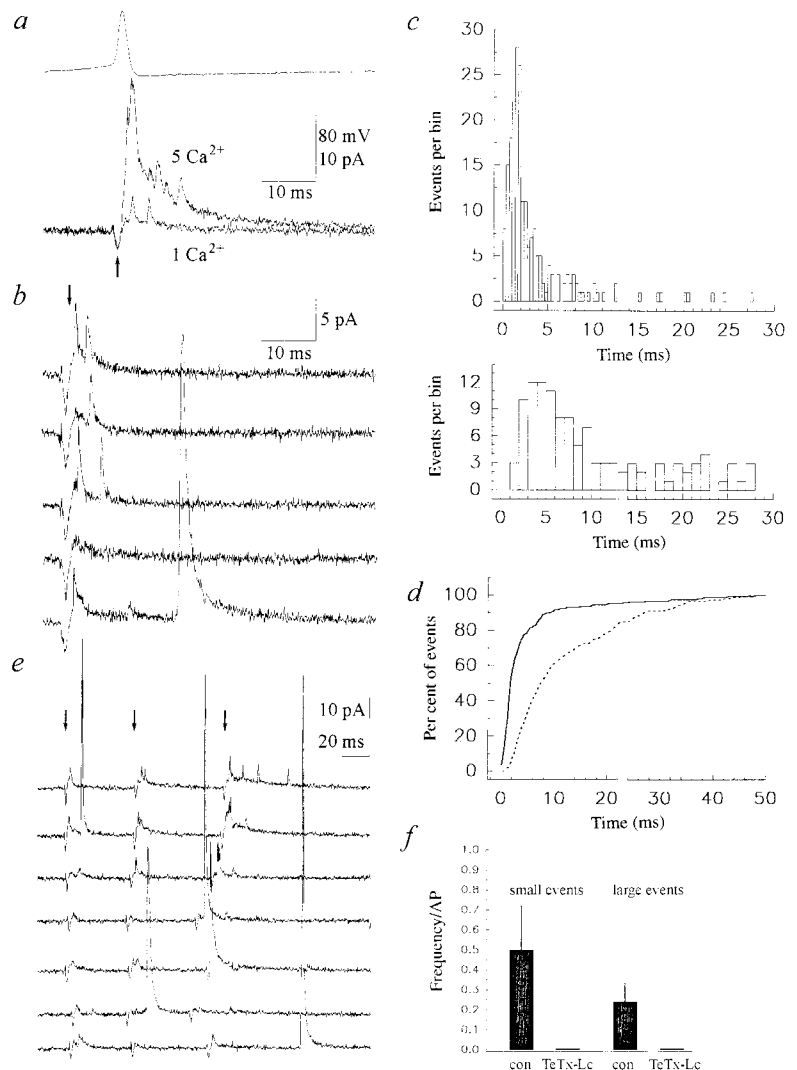
To quantify the amount of serotonin released per vesicle, we determined how many electrons are transferred per oxidized serotonin molecule (see Fig. 2c for details). We found that one serotonin molecule contributes 4 electrons on average. A single SSV thus releases on average $4,700 \pm 150$ serotonin molecules and the intravesicular concentration of serotonin is 270 mM. This amount of serotonin per SSV is half the number of acetylcholine molecules in frog SSV, as estimated by iontophoretic application of transmitter at the neuromuscular synapse¹⁰. LDCV release, on average, 16-fold more serotonin, ranging from 15,000 to 300,000 molecules. Given the size heterogeneity¹¹ of the LDCV (see also Fig. 2d), it follows that serotonin may be stored at a concentration comparable to that in SSV.

Exocytosis of SSV is initiated by a rapid upstroke of the transmitter signal with a mean 50–90% rise time of 90 μ s (median, 50 μ s). In fact, the rise time of many small events was indistinguishable from the step response of our measuring system (Fig. 3a). Thus, it can be estimated that the actual rise of the transmitter signal at the plasma membrane and diffusion of serotonin to the detector occur in less than 60 μ s. This suggests that the opening of a preassembled fusion pore initiates transmitter discharge from SSV in neurons.

In non-neuronal cells, amperometrically recorded exocytotic events are sometimes preceded by a small 'foot' signal^{2,3} which reflects the diffusion of transmitter through a slowly dilating fusion pore formed at the onset of exocytosis¹². In the neurons

FIG. 1 Electrochemical detection of synaptic vesicle exocytosis evoked by action potentials. **a**, An action potential (generated by depolarizing current injections using an intracellular microelectrode, top) in 5 mM Ca^{2+} evokes a complex current transient (bottom) with spike-like current deflections. In 1 mM Ca^{2+} , we observed individual events with an amplitude similar to that of the current deflections seen under high Ca^{2+} . Thus, these spikes probably represent single exocytotic events that occur close to the sensing surface of the carbon fibre. The electrical artefact (arrow) in the current trace is caused by capacity coupling between the microelectrode and the carbon fibre. **b**, Representative current transients evoked by single action potentials in 1 mM Ca^{2+} (stimulus interval 30 s). Small events follow the peak of the action potential (arrow) with a short delay. Occasionally, we observed large events that occurred with a longer delay (bottom trace); second trace from bottom shows a 'failure' (sweep without a response). **c**, Latency histograms of small and large current transients (latency defined as time interval between the peak of the action potential and the onset of the current transient, first 30 ms shown). The release probability of the small events (top; bin width 333 μ s, 8 cells, 380 APs, 220 events) peaks at 1.6 ms and sharply declines within the next 5 ms. Some events occurred with no measurable delay after the peak of the action potential. The average rate of spontaneously occurring small events (0.04 s^{-1}) was too low to affect the shape of the histogram. Large events (lower panel; bin width 1 ms, 34 cells, 3,600 APs, 140 events) occurred with a minimum delay of 1 ms after the peak of the action potential. The peak of the histogram is at 4–5 ms, followed by a 'tail' of longer-latency responses. The average rate of spontaneously occurring large events was 0.05 s^{-1} , contributing ~6 events. This does not change the major characteristics of the histogram. **d**, Integrated latency histograms (shown under **c**) normalized to the total number of events occurring during the first 50 ms after the action potential. By comparing the maximum slopes of the integrated latency histograms we estimate that the maximum secretion rate of small events (solid line) after single action potentials is 3.5 times higher than that of large events (dashed line). **e**, Current transients evoked by trains of 3 action potentials (successive sweeps, 1-s intervals) in 1 mM Ca^{2+} . Individual action potentials are indicated by arrows for the first sweep. The frequency of large events is higher (0.20 ± 0.02 events per AP, $n=8$) than after stimulation with single action potentials (0.05 ± 0.04 events per AP, $n=7$; not shown). **f**, The frequency of small events evoked by single action potentials was greatly reduced after microinjection of TeTx-Lc (0.5 mg ml^{-1} ; $n=4$) compared to control cells (con) injected with carrier solution ($n=6$). The same effect was seen for large events evoked by trains of action potentials.

METHODS. Retzius cells of the leech (*Hirudo medicinalis*) were isolated from de-sheathed ganglia as described¹, plated on polyornithine-coated dishes, and used for recordings on days 2–5 in culture. Microelectrodes (resistance 30–50 M Ω) were back-filled with 3 M potassium acetate, 100 mM KCl and were connected to a conventional bridge amplifier. Extracellular solution (Ringer) in mM: 130 NaCl, 4 KCl, 1 or 5 CaCl_2 , 1 MgCl_2 , 10 Na-HEPES, 70 glucose, pH 7.3. Carbon fibre electrodes (6.0 μ m diameter) were prepared as described², back-filled with 3 M



KCl solution and connected to a List EPC-7 amplifier. The voltage of the electrode was set to +650 mV. Spontaneously occurring signals were recorded in the event-detecting mode. The amperometric current signals were filtered in two stages 13 kHz and 10 kHz, digitized at a rate of 25–100 kHz, stored on a personal computer and analysed with the program AutesP. Data were fitted using a least-squares criterion. Tetanus-toxin light chain was pressure-injected through a microelectrode 90 min before the experiment. Carrier solution contained (in mM) 35 NaCl, 10 Na-HEPES, pH 7.3. The injected volume (2–5% of the cell volume) was estimated by subsequent test injections into an oil drop. Experiments were done at room temperature (19–25 °C). Measurements are given as mean $\pm$ s.e.m. unless indicated otherwise.

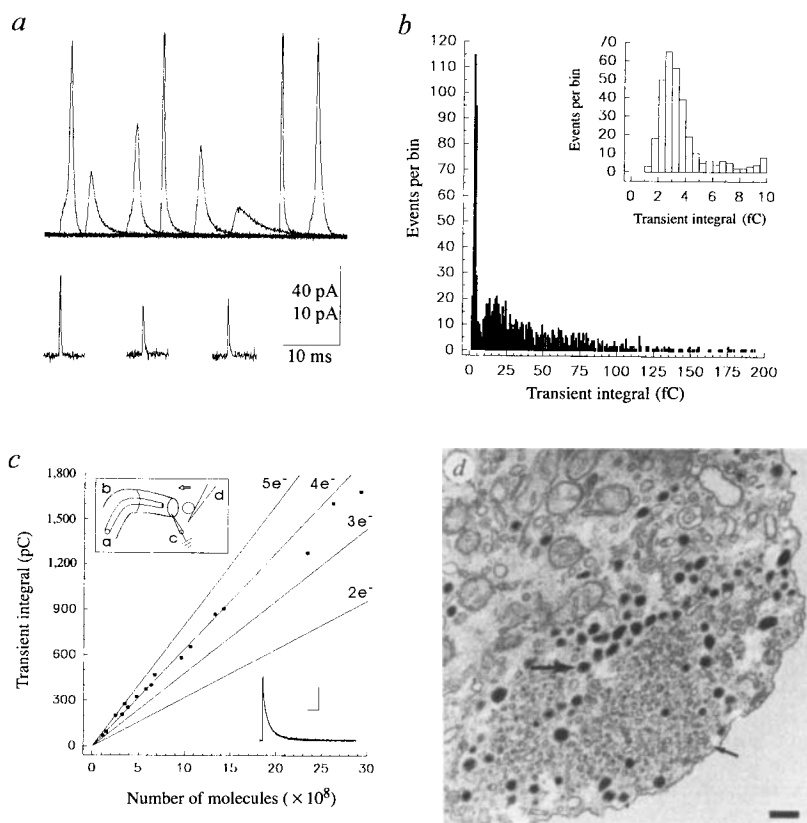
used in our studies, one third of the large events were preceded by 'foot' signals, which last on average 540 μ s (Fig. 3b). These 'foot' signals were often initiated by a stepwise increase of the current signal, with a mean 50–90% rise time of 110 μ s and a mean amplitude of 7.5 pA, similar to that seen in small events. This suggests that the similar magnitude of the transmitter signal initiating both events may arise from the first opening of similar-sized fusion pores. Occasionally the upstroke of the signal-initiating transmitter discharge from LDCV was not followed by an increase in amplitude, suggesting that the expansion of the pore was hindered (Fig. 3c). These events show a long-lasting and slowly fading response, as expected for transmitter release from a large synaptic vesicle through a narrow fusion pore. Hence rapid discharge of transmitter from LDCV probably depends on the expansion of the fusion pore during the first milliseconds.

Calculations by Almers *et al.*¹³ predict that SSV discharge their content with a time constant of only 250 μ s through an undilated fusion pore (300 pS; initial conductance of fusion pores in mast cells¹³). The mean time constant of decay of small events was 360 μ s (median 260 μ s; Fig. 3d). Thus, dilation of the initially formed fusion pore is not necessary to explain the rapid time course of transmitter release from SSV.

In summary, our experiments have directly measured transmitter release from individual synaptic vesicles in neurons. The

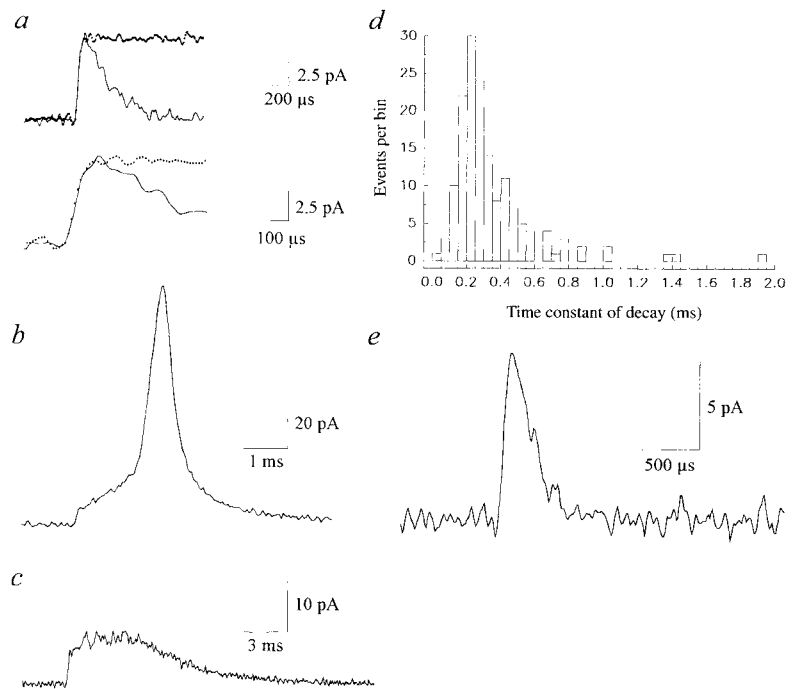
different properties of small and large events with respect to charge, amplitude and kinetics, together with our electron microscope data strongly suggest that these signals are caused by exocytosis of SSV and LDCV, respectively. Exocytosis of SSV occurred more rapidly and more frequently after single action potentials than exocytosis of LDCV. Exocytosis in neurons is regulated by the intracellular calcium level ($[Ca^{2+}]_i$) beneath the plasma membrane where steep Ca^{2+} gradients exist^{14,15}. Based on the different spatial organization of SSV and LDCV in our neurons, it might be assumed that exocytosis of LDCV is less well coupled to action potentials than that of SSV because Ca^{2+} has to diffuse a greater distance to trigger secretion of the more randomly positioned LDCV. However, differences in the exocytotic speed could also be due to different rates of the fusion processes controlling secretion of SSV and LDCV, as suggested by studies showing that the maximum secretion rate of SSV in bipolar neurons is higher than that of granules in chromaffin cells¹⁶. Exocytosis of both types of vesicles seems to be initiated by an abrupt opening of a preassembled fusion pore. The kinetic properties of this mechanism resemble that of fusion pores that form during exocytosis of mast cell vesicles¹⁷. Whereas rapid transmitter release from LDCV depends on the expansion of the pore during the first milliseconds, SSV discharge their content on a submillisecond timescale, which could be accomplished

FIG. 2 a, Spontaneously occurring exocytosis of SSV and LDCV. Large events (upper panel, eight superimposed traces) and small events (lower panel, three single traces) occurred at a mean rate of 0.05 Hz and 0.04 Hz ($n=6$), respectively. The rate of spontaneously occurring small and large events was greatly diminished after microinjection of tetanus-toxin light chain (0.005 Hz, $n=5$, data not shown). Note the different amplitude scales for the two panels. b, Charge distribution of current transients in femtocoulombs (fC) plotted from 1080 events (7 cells) shows two clearly separated peaks (bin width 1 fC). The charge of small events was confined to a narrow range (1–8 fC) consistent with the homogeneous size distribution of SSV in Retzius cells¹¹ (diameter, 37.8 ± 0.7 nm). The charge of large events varied over a wider range (10–200 fC) as is anticipated for the more heterogeneously sized LDCV (diameter, 55–150 nm); inset: charge distribution of small events at a bin width of 0.5 fC; mean 3.6 ± 0.1 fC (median, 3.0 fC; range <8 fC). Charge and frequency of large events make it unlikely that large spikes are due to compound fusion of SSV. c, Determination of the number of electrons transferred during oxidation of serotonin. A carbon fibre (a, upper inset) was inserted into a glass capillary tube (b) which was filled from the tip with Ringer solution (volume ~ 1.6 nl). The set-up was covered with oil and a microelectrode (c, with a broken tip, filled with Ringer solution) was manipulated into the tip of the glass capillary, serving as a reference electrode. Droplets were produced by pressure-ejection of Ringer solution (containing 30 μ M serotonin) from a separate microelectrode (d) and their diameter was measured. Upon contact of the droplet with Ringer–oil interface, it detached itself from the electrode and discharged its content into the reaction chamber. The integral of the transient oxidation current (lower inset) can be directly related to calculated number of applied molecules by Faraday's law: $Q = Mez$, where M is the number of molecules, e is the elementary charge 1.6×10^{-19} coul and z is the number of moles of electrons transferred per mol of serotonin reacted. Straight lines represent the prediction by this relationship for different z -values. The integral of the oxidation current increased linearly with the amount of applied transmitter and correlates well with the prediction of 4 electrons transferred per oxidized serotonin molecule, agreeing with the mechanism proposed for the dominant electrochemical oxidation reaction¹⁹ (lower inset, calibration, vertical 50 pA, horizontal 5 s). d, Electron micrograph of synaptic vesicles located at the tip of the



axon stump of a cultured Retzius cell. Retzius cells (see also refs 11, 20) contain, like other serotonergic neurons²¹, SSV and LDCV. SSV (small arrow) are clustered next to the plasma membrane. LDCV (large arrow) surround these clusters and are scattered throughout the cytoplasm. No clusters of SSV, but only LDCV were observed in the cell soma, agreeing well with our amperometric results that only large events were measured at somatic recording sites (data not shown). This makes the possibility unlikely that small events are due to transient fusion of LDCV. For electron microscopy, cells were fixed and processed as described¹¹. Calibration, 0.2 μ m.

FIG. 3 Kinetic characteristics of transmitter discharge from SSV and LDCV. *a*, Initiation of transmitter discharge from SSV. Ten individual small events were averaged after alignment with respect to the midpoint of their rising phase. The same procedure was followed to obtain the instrument's step response, using records from capacitively injected current steps (amplitude 8 pA). The step response (dotted line) is superimposed on the rising phase of the small event (continuous line) after amplitude scaling. The 10–90% rise time of the step response was 60–70 μ s. The time course of the rising phase of the small event is indistinguishable from the step response of the measuring system (difference less than 10 μ s, maximum digitizing rate). *b*, 'Foot' signals often precede the main phase of transmitter discharge from LDCV. The foot had a duration of 540 ± 40 μ s and a maximal amplitude of 10 ± 1 pA. The integral of the foot signal reflects on average $4 \pm 0.5\%$ of the event's total integral ($n = 230$). Half of the foot signals were initiated by a stepwise increase of the current signal with an amplitude of 7.5 ± 0.7 pA (median, 4.5 pA) and a 50–90% rise time of 110 ± 10 μ s (median, 60 μ s; $n = 110$). Owing to the low rate of spontaneously occurring events (0.05 Hz) it is extremely unlikely that the initial step results from an accidental superimposition of small and large events. Large events initiated by such a step presumably arise from vesicles opening closest to the detector, in agreement with their short half-width (1.1 ± 0.8 ms, one third of the average half-width of large events; Table 1). *c*, Recording of transmitter release from an LDCV (charge 75 fC), which is initiated by a rapid stepwise increase of the transmitter signal but is not followed by a large amperometric spike. These events had an average half-width of 8 ± 11 ms ($n = 45$, 5% of all large events). Note the fast fluctuations of the current trace, which clearly exceed the baseline noise and might represent flickering of the vesicle fusion pore. Our experiments do not exclude the possibility that other mechanisms, like the dissolution of a storage matrix (as suggested by experiments on mast cells granules¹²), may also influence the kinetics and the magnitude of transmitter discharge from SSV and LDCV. *d*, Frequency distribution of the time constant of decay of spontaneously occurring small events. Decays of small events were fitted by single exponentials. The



median was 260 μ s, the mean was 360 ± 30 μ s ($n = 150$). LDCV release their content with a time constant of 2.3 ± 0.2 ms (median, 1.3 ms; $n = 140$). It should be noted that our measurements are limited by the time of diffusion of transmitter to the surface of the carbon fibre, hence reflecting an upper limit for the speed of transmitter discharge. *e*, Exemplary recording of transmitter discharge from a SSV at high resolution. The vesicle released 4,200 transmitter molecules with a time constant of 160 μ s.

through an undiluted fusion pore. Although the fate of a SSV after transmitter discharge remains unclear, it is possible that SSV are recycled without ever undergoing full fusion, which would be advantageous for rapid and selective retrieval of vesicle membrane¹⁸. □

Received 26 April; accepted 5 July 1995.

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ACKNOWLEDGEMENTS. We thank F. Sigworth, P. Hanson, H. Otto, E. Chapman and R. Ossig for their suggestions regarding the manuscript; S. Engers for cultured neurons and for technical assistance; R. Chow for providing information on the carbon fibre technique; L. Daniell and P. Steen for help with the EM part of this study; and H. Niemann for tetanus-toxin light chain. This work was supported by a Max-Planck-Society fellowship to D.B.

Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID)

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SEVERE combined immune deficiency (SCID) represents a heterogeneous group of hereditary diseases. Mutations in the common γ -chain (γ^c), which is part of several cytokine receptors including those for interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-15, are responsible for X-linked SCID^{1,2}, which is usually associated with a lack of circulating T cells and the presence of B lymphocytes

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($T^- B^+$ SCID). The gene(s) responsible for autosomal recessive $T^- B^+$ SCID is still unknown. The Jak-3 protein kinase^{3,4} has been found to associate with the γ^c -chain-containing cytokine receptors⁴⁻⁹. Therefore Jak-3 or other STAT proteins with which it interacts^{10,11} are candidate genes for autosomal recessive $T^- B^+$ SCID⁷. Here we investigate two unrelated $T^- B^+$ SCID patients (both from consanguineous parents) who have homozygous mutations in the gene for Jak-3. One patient carries a mutation (Tyr100→Cys) in a conserved tyrosine residue in the JH7 domain of Jak-3 which is absent in more than 150 investigated chromosomes. The other patient carries a homozygous 151-base-pair deletion in the kinase-like domain, leading to a frameshift and premature termination. Both mutations resulted in markedly reduced levels of Jak-3. These findings show that abnormalities in the Jak/STAT signalling pathway can account for SCID in humans.

To facilitate the detection of putative Jak-3 mutations, we selected two unrelated male $T^- B^+$ SCID patients, both with consanguineous parents, in which homozygous abnormalities could be expected. Despite an increased proportion of B lymphocytes in peripheral blood cells (the CD19⁺ lymphocytes being 94% of circulating mononuclear cells in subject G.M. and 90% of circulating mononuclear cells in subject C.M.), both patients showed clear signs of impairment of B-cell function *in vivo*. At 3 months of age, before treatment with intravenous immunoglobulins, both patients were severely hypogammaglobulinaemic (patient G.M., IgG 1,000 mg l⁻¹, IgA <66 mg l⁻¹, IgM 300 mg l⁻¹; patient C.M., IgG 1,300 mg l⁻¹, IgA <66 mg l⁻¹, IgM 150 mg l⁻¹), and both lacked isoagglutinins.

Analysis of the entire γ^c gene sequence in patient G.M. showed that it was normal, ruling out an X-linked origin of the disorder. To investigate whether a deficiency in Jak-3 might explain the immunological defect of this patient, we sequenced reverse transcription-polymerase chain reaction (RT-PCR) amplification products obtained from Jak-3 messenger RNA extracted from an Epstein-Barr virus (EBV)-transformed B-cell line, which appeared normal in size (data not shown).

FIG. 2 Restriction analysis of the genomic region around the mutation of patient G.M. *a*, Electrophoresis of PCR products obtained by DNA amplification with primers 282F and In-2. Lane 1, patient G.M.; lane 2, mother of patient G.M.; lanes 3–16, normal controls. *b*, Schematic representation of the *RsaI* restriction sites in normal and mutated DNA around nucleotide 394. The arrowheads indicate *RsaI* sites; the white one indicates the site eliminated by the A→G mutation at nucleotide 394 in the abnormal allele from patient G.M. family members. This results in a 141-bp band instead of the 99- and 42-bp bands, in addition to two other bands of 30 and 13 (the latter is not visible in the gel).

METHODS. For *RsaI* digestion analysis, PCR was performed on DNA extracted from peripheral blood cells of the patient, his mother and normal controls, with the primer pair 282F/In-2 (282F: ATCTGCTGTG-TACACT; In-2: CCTCGAATGCAAGGAGATG), the latter being located in the second intron. Amplification conditions were as described for Fig. 1. The products were digested according to the manufacturer's instructions and separated on an 8% polyacrylamide gel.

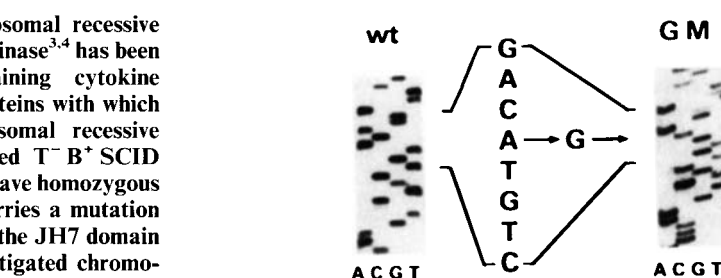


FIG. 1 Tyr100→Cys mutation in the Jak-3 gene of patient G.M. with autosomal SCID. The sequence of the region around nucleotide 394 of the Jak-3 sequence (Accession Number U09607), demonstrating an A→G transition (arrow) at this nucleotide, is shown.

METHODS. RNA was extracted from an EBV-transformed lymphoblastoid cell line obtained from peripheral blood cells of patient GM, according to standard techniques¹⁸. RT-PCR was performed with primer pair 96F/600R (96F: ATGGCACCTCCAAGTGAAG; 600R: CCAACACGGCCAGGCT-GAGA). The numbers refer to the nucleotide position in the complementary DNA Jak-3 sequence, and F means forward and R reverse. The amplification conditions were as follows: 30 cycles of denaturation at 94 °C, annealing at 59 °C and extension at 72 °C, each for 30 s. The resulting PCR product was cloned in TA cloning vector (Invitrogen) and sequenced by dideoxynucleotide chain termination method using the Sequenase kit (USB), as described previously¹⁹.

The same mutation, an A→G transition at nucleotide 394, which causes a tyrosine-to-cysteine substitution at amino acid 100 (Tyr100→Cys), was found in six clones from patient G.M. (Fig. 1). This mutation results in the disappearance of an *RsaI* site. To exclude a rare polymorphism, we analysed more than 150 chromosomes. As our investigation of the corresponding genomic region showed the presence of an intron at nucleotide 403 of the Jak-3 transcript, an upstream exonic primer (282F) and a downstream intronic primer (In-2) were used to amplify a 184-bp-long PCR product from genomic DNA, encompassing the mutated nucleotide.

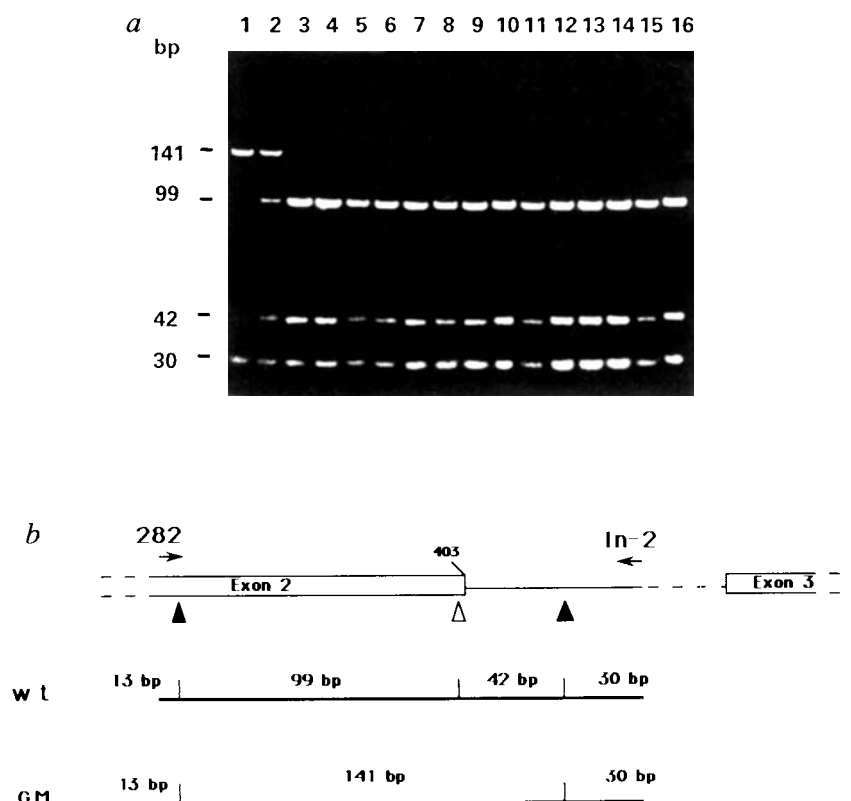
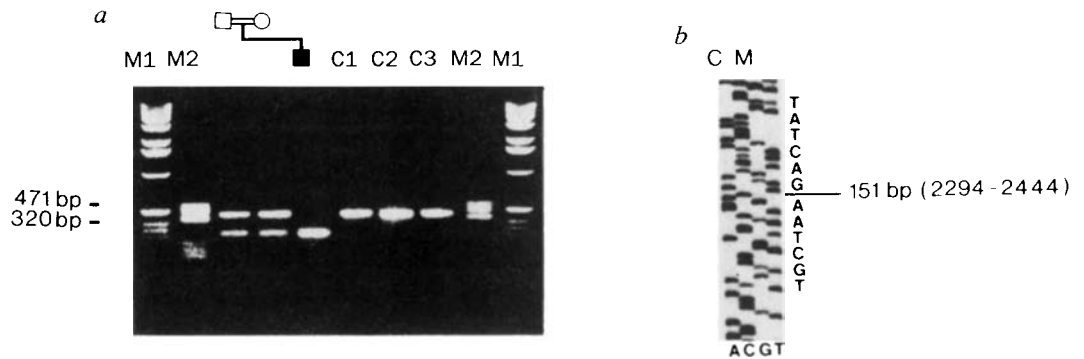


FIG. 3 Deletion of the region between nucleotides 2,294 and 2,444 in patient C.M. *a*, Electrophoresis of RT-PCR products obtained by mRNA amplification with primers 2130F and 2600R. M1, 1-kb marker; M2, marker V (Boehringer); lane 1 (white square), father of patient C.M.; lane 2 (circle), mother of C.M.; lane 3 (black square), patient C.M.; lanes 4–6 (C1–C3), normal controls. *b*, Sequence of the region surrounding the deletion, showing the absence of a region of 151 bp from nucleotides 2,294 and 2,444 included.

METHODS. mRNA extraction, subcloning in TA vector and sequencing was performed as described for Fig. 1. The primer pair used was:



2130F/2600R (2130: TTAAGCCTGGAGATGCTCAC; 2600R: GCTGCCAAAGTTGCCCTTGC). Conditions for the RT-PCR were as described for Fig. 1, except that the annealing step was at 60 °C.

RsaI digestion of specific PCR products from 154 independent chromosomes demonstrated, in all cases, the presence of the expected bands of 99, 42, 30 and 13 bp. In contrast, in patient G.M. the bands of 99 and 42 bp were replaced by one band of 141 bp (Fig. 2*a, b*). These data are in keeping with homozygosity for the mutation. Accordingly, in addition to the four normal digestion products, the abnormal 141-bp band was also present in the mother of patient G.M., showing her to be heterozygous for the mutation (DNA from the father was not available as he is deceased).

The amplification of the Jak-3 transcript in the second patient (C.M.) revealed a smaller-than-expected band when the amplification was performed with exonic primers 2130F and 2600R. As shown in Fig. 3*a*, the expected 471-bp band was detected in normal controls whereas only a shorter band was seen in patient C.M., suggesting that the patient was homozygous for a deletion in this transcript region. Accordingly, from both father and mother a shorter PCR product (of the same size as that found in the proband) was present in addition to the normal one. Sequencing of 5 clones showed that all the clones of patient C.M. bore a deletion of 151 nucleotides, from nucleotides 2,294 to 2,444 (inclusive), in agreement with the RT-PCR analysis (Fig. 3*b*). This deletion causes a shift in the reading frame after amino acid 733 (in the final portion of the kinase-like domain), leading to premature termination (a TAG stop codon is formed at nucleotides 2,626–2,628). It seems highly unlikely that the deleted PCR product represents a normal alternative transcript as we have not seen it previously described and it was never present in our controls, but it was present at the homozygous level in the proband and at the heterozygous levels in his parents. In addition, a normal-sized PCR product was absent in the proband.

To analyse the consequences of the patients' mutations of the Jak-3 gene on the function of Jak-3 protein, we first immunoblotted whole-cell lysates with anti-Jak-3 antibody³ to determine the level of expression of the mutated protein. As shown in Fig. 4 (top), EBV-transformed normal B cells (lane 2) had levels of Jak-3 protein comparable to those seen in the NK3.3 cell line (lane 1), which expresses high levels of Jak-3 (refs 4, 5). In sharp contrast, neither patient expressed substantial amounts of Jak-3 protein; patient G.M. had barely detectable Jak-3 protein (lane 3), and patient C.M. had too little to be detected (lane 4). Thus both of these mutations lead to marked reduction in the steady-state levels of Jak-3. To ascertain equal loading of protein from each of these samples, the filter was stripped and reprobbed with anti-Jak-1 antibody. As shown in Fig. 4 (bottom), unlike Jak-3, Jak-1 was expressed at roughly comparable levels in all of the cells. Thus it appears that the mutant Jak-3 alleles in both patients generated protein products that are poorly expressed.

In future studies it will be important to determine the mechanism responsible for the poor expression of these mutants proteins. However, this lack of expression of Jak-3 protein is likely to be pathogenetically responsible for the immune deficiency in these patients.

In contrast to other Jaks that are ubiquitously expressed, Jak-3 has been shown to have limited tissue expression^{3, 5, 9}. Con-

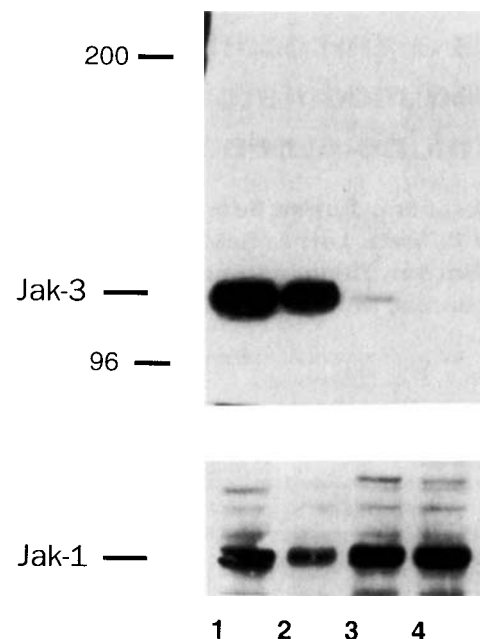


FIG. 4 Markedly reduced levels of Jak-3 protein in EBV-transformed B cells from patients with Jak-3 mutations. Lysates from the NK3.3 cell line (lane 1), EBV-transformed B cells from a normal individual (lane 2), and EBV-transformed B cells from patient G.M. (Tyr100→Cys mutation, lane 3) and patient C.M. (deletion of 2,294–2,444, lane 4) were electrophoresed and immunoblotted for Jak-3 expression (top). The filter was stripped and reprobbed for Jak-1 expression (bottom). Jak-3 expression was found to be greatly reduced in patient cells, whereas the level of Jak-1 expression was similar for all of the cell lines.

METHODS. Cells (5×10^6) were lysed as described previously⁵. Post-nuclear supernatants were assayed for protein concentration (BCA reagent, Biorad), and 100 µg of each sample was electrophoresed on an 8% SDS-polyacrylamide gel, transferred to Immobilon and immunoblotted with anti-Jak-3 (top) antibody³ and developed by enhanced chemiluminescence (ECL, Amersham). The filter was stripped according to the manufacturer's recommendations and reblotted with anti-Jak-1 (bottom) antibody (Transduction Labs, Lexington, KY).

sistent with these previous observations is the present demonstration that mutation of Jak-3 does not appear to have developmental consequences other than the effects on the immune system, because, apart from the immune deficiency, the two patients are normal. Moreover, since bone-marrow transplantation, patient G.M. has developed normally (patient C.M. has not yet undergone transplantation). This indicates that the principal functions of Jak-3 relate to lymphoid development, and it is dispensable for the development of other systems.

Mutation of the γ_c gene in humans results in $T^- B^+$ X-linked SCID^{1,2}, and knocking out the corresponding gene in mice heavily affects both T- and B-lymphoid development^{12,13}. The detection of Jak-3 mutations in two patients with $T^- B^+$ SCID confirms that in humans the γ_c chain/Jak/STAT signalling path-

way is critical to early T-cell, but not B-cell, development. In addition, both patients examined here showed a lack of circulating natural killer (NK) cells (as detected by CD16 monoclonal antibody). Lack of circulating NK cells is not unique to Jak-3-deficient SCID patients, having also been reported in X-linked SCID¹⁴. Further, carrier females of X-linked SCID exhibit a non-random pattern of X-chromosome inactivation in NK cells¹⁵, as well as in T and B lymphocytes^{16,17}, indicating that mutations of the γ_c gene interfere with proliferation/differentiation of all these lymphoid lineages. As the γ_c chain and Jak-3 are elements of the same signal-transducing pathway and are both expressed in NK cells, it is tempting to speculate that the integrity of this pathway is strictly required for NK-cell development. □

Received 2 June; accepted 11 August 1995.

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ACKNOWLEDGEMENTS. P.M. and A.V. contributed equally to this work. We thank R. Dulbecco and D. Nelson for suggestions; L. Rossi Bernardi for encouragement; M. Littardi, D. Strina and L. Susani for technical assistance; S. Bevan for typing the manuscript; and J. Kornbuth for promoting the NK3.3 cell line. This work was supported by grants from CNR (P.F. Ingegneria Genetica to L.N. and P.V., and ACRO to A.V.), Biomed 1 (PL 1321 to L.N.), AIRC (to A.V.) and Telethon Italy (to A.U. and P.V.).

MIF as a glucocorticoid-induced modulator of cytokine production

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GLUCOCORTICOID hormones are important for vital functions and act to modulate inflammatory and immune responses^{1,2}. Yet, in contrast to other hormonal systems, no endogenous mediators have been identified that can directly counter-regulate their potent anti-inflammatory and immunosuppressive properties. Recent investigations of the protein macrophage migration inhibitory factor (MIF), which was discovered originally to be a T-lymphocyte-derived factor^{3,4}, have established it to be a pro-inflammatory pituitary and macrophage cytokine and a critical mediator of septic shock^{5–7}. Here we report the unexpected finding that low concentrations of glucocorticoids induce rather than inhibit MIF production from macrophages. MIF then acts to override glucocorticoid-mediated inhibition of cytokine secretion by lipopolysaccharide (LPS)-stimulated monocytes and to overcome glucocorticoid protection against lethal endotoxaemia. These observations identify a unique counter-regulatory system that functions to control inflammatory and immune responses.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an essential feature of the systemic stress response, and leads ultimately to an increase in circulating levels of glucocorticoids, which are essential for modulating the host response to infection and tissue invasion^{1,2,8}. Because glucocorticoids are potent inhibitors of cytokine production^{2,9–13}, we examined their effect

on the production of MIF by LPS-activated macrophages *in vitro*. In sharp contrast to expectations, glucocorticoids alone were found to induce rather than inhibit MIF production. MIF secretion was induced by low concentrations of dexamethasone or hydrocortisone (cortisol), the principal glucocorticoid hormone in humans, and was detectable in culture medium 3 hours after glucocorticoid stimulation (Fig. 1). Glucocorticoid-induced macrophage MIF secretion showed a bell-shaped dose-response curve when assessed by a sensitive, MIF-specific enzyme-linked immunosorbent assay (ELISA). MIF release was triggered by concentrations of dexamethasone as low as 10^{-16} M, peaked at 10^{-14} to 10^{-12} M, and decreased at higher concentrations (10^{-8} to 10^{-6} M) (Fig. 1). A similar profile of glucocorticoid-induced MIF secretion was observed in primary mouse

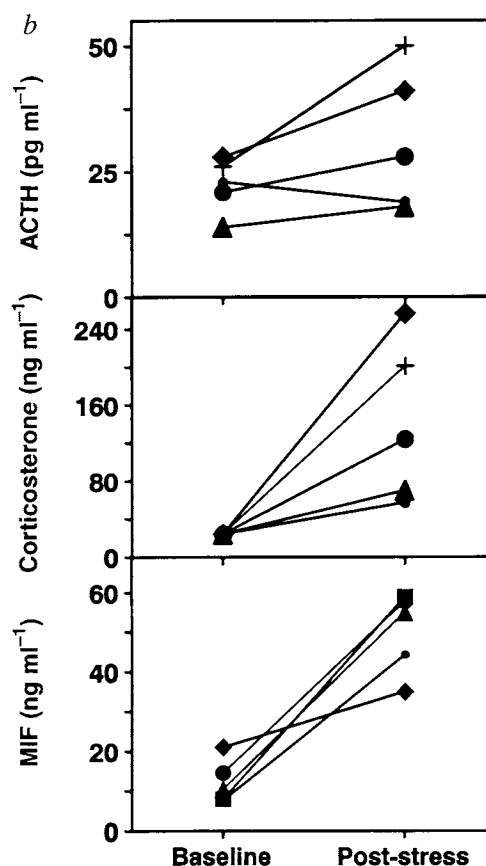
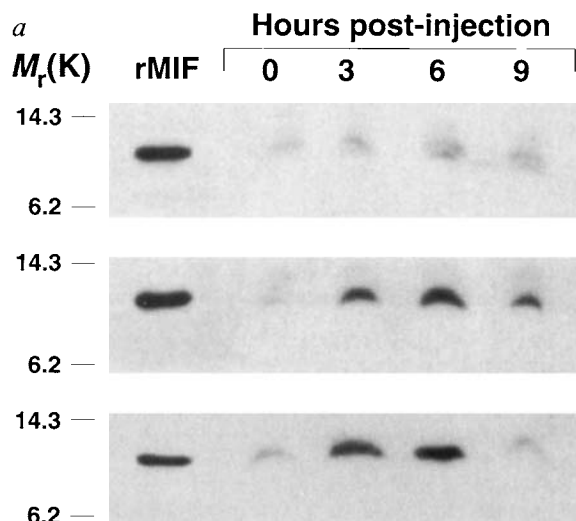
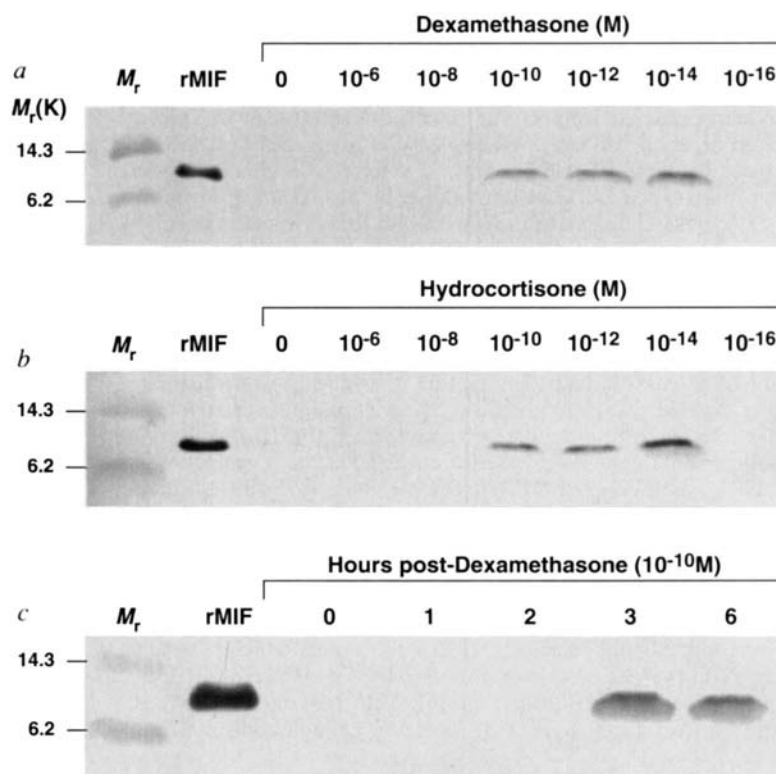
FIG. 2 Glucocorticoid-induced MIF production *in vivo*. a, Western blot analyses of serum MIF in vehicle-injected control rats (top) and rats injected with 1 mg per kg (middle) and 10 mg per kg (bottom) dexamethasone.

METHODS. Female Sprague-Dawley rats (250–300 g) were prepared surgically with an indwelling catheter in the jugular vein 5–7 days before the experiment. The catheter was externalized via a headset and connected to a tethered line which allowed injection of drug and collection of blood with minimal disturbance to the animals. Rats were housed individually, had free access to food and water, and were maintained on a 12-h light/12-h dark cycle (lights on at 7:00). All experiments commenced at 8:00. Dexamethasone, diluted in sterile saline, was injected intravenously (0.1–20 mg per kg) when all rats were resting and had been undisturbed for at least 1 h. The injected materials were determined previously to be free of endotoxin contamination by the chromogenic *Limulus* amoebocyte assay (BioWhittaker, Walkersville, MD). Blood was withdrawn via the catheter at 0 (baseline), 1.5, 3.0, 4.5, 6.0, 7.5 and 9.0 h after injection of saline or dexamethasone and analysed for MIF content by western blotting or ELISA. For western blotting, portions of 6 μ l serum were analysed as described in Fig. 1, and the MIF bands visualized by a chemiluminescence reaction (ECL; Amersham) and exposure on X-OMAT-XAR film (Eastman Kodak, Rochester, NY). As a reference standard, rMIF was electrophoresed and transferred in lane 1. By ELISA, the serum concentration of MIF in resting, unstressed animals (serum-corticosterone concentrations <25 ng ml⁻¹) was 15.9 ± 6.5 ng ml⁻¹ (mean $\pm$ s.d. of 14 rats) and

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FIG. 1 Glucocorticoid-induced MIF secretion by macrophages. Western blot analysis of MIF secretion by RAW 264.7 macrophages incubated for 12 h with dexamethasone (a) or hydrocortisone (b) at the indicated concentrations. Also shown is a time-course analysis of MIF secretion by RAW 264.7 cells incubated with dexamethasone (10^{-10} M) (c).

METHODS. RAW 264.7 macrophages (3×10^6 cells) were cultured in RPMI/1% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and stimulated with dexamethasone or hydrocortisone (Sigma, St Louis, MO). At intervals, the cell-conditioned media were removed and concentrated tenfold by membrane filtration (cut-off at M_r 10K). The retained proteins were resolved by electrophoresis through 18% SDS polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated with rabbit polyclonal anti-murine rMIF serum (1:1,000), and then with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000) (Pierce, Rockford, IL). MIF was visualized by development with chloronaphthol/ H_2O_2 (refs 5, 6). rMIF (10 ng) was electrophoresed and transferred as a standard. M_r denotes relative molecular mass standards. MIF concentrations in cell-conditioned media were quantified by a sensitive, MIF-specific ELISA following a protocol to be described elsewhere (C.N.M. et al., in preparation). Samples were analysed in triplicate and the results expressed as mean $\pm$ s.e.m. of two separate experiments. MIF concentrations were 308 ± 53 pg ml $^{-1}$ (control cells) and 668 ± 220 , 331 ± 96 , $1,911 \pm 60$, $2,174 \pm 143$, $3,271 \pm 694$ and $2,025 \pm 336$ pg ml $^{-1}$ in supernatants of cells induced with 10^{-6} , 10^{-8} , 10^{-10} , 10^{-12} , 10^{-14} and 10^{-16} M of dexamethasone, respectively.



appears to reflect the normal, circulating levels of MIF. Peak serum concentrations of MIF were in the range 35–40, 45–80 and 100–120 ng ml $^{-1}$ in rats injected with 1, 10 and 20 mg per kg dexamethasone, respectively. **b**, Serum concentrations of ACTH, corticosterone and MIF after stress-induced activation of the HPA axis. Blood was obtained from five catheterized rats at baseline and 1.5, 3.0, 4.5, 6.0 and 9.0 h after handling-induced stress²⁴. ACTH and corticosterone were measured by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA) and MIF was measured by ELISA. Serum levels (mean $\pm$ s.d.) of MIF increased from 12.4 ± 5.6 ng ml $^{-1}$ (baseline) to 50.2 ± 10.3 ng ml $^{-1}$ within 3 to 4.5 h post-stress ($P=0.004$). During the same period of time, ACTH and corticosterone levels increased from 22.4 ± 5.4 to 31.2 ± 14.0 pg ml $^{-1}$ ($P=0.13$), and from <25 to 141.8 ± 85.8 ng ml $^{-1}$ ($P=0.04$), respectively. Each symbol represents the values obtained from one experimental animal.

monocytes/macrophages and in T lymphocytes (data not shown).

To investigate glucocorticoid-induced MIF production *in vivo*, we performed both western blotting and ELISA analyses of sera obtained from rats injected intravenously with various concentrations of dexamethasone (0.1–20 mg per kg). Glucocorticoid administration produced a time- and concentration-dependent increase in serum MIF (Fig. 2a). High endogenous glucocorticoid concentrations were also found to affect circulating MIF levels. As shown in Fig. 2b, stress-induced activation of the HPA axis in rats produced an elevation in adrenocorticotrophic hormone (ACTH) and corticosterone levels that was associated with a concomitant rise in the serum levels of MIF.

The unanticipated and at first glance paradoxical finding that glucocorticoids induce the secretion of a specific, pro-inflammatory cytokine led us to postulate that MIF might act to counter-regulate the anti-inflammatory activities of glucocorticoids. To address this hypothesis, we examined the effect of exogenously added, recombinant MIF (rMIF) on glucocorticoid inhibition of cytokine production *in vitro*, and on endotoxin-induced lethality *in vivo*. rMIF was found to override, in a dose-dependent fashion, glucocorticoid inhibition of tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and IL-8 secretion by LPS-stimulated human monocytes (Fig. 3a). The ability of MIF to overcome the inhibition of TNF- α secretion varied with the concentration of glucocorticoid and decreased with increasing dexamethasone concentration (Fig. 3b). That MIF is not induced, and has a reduced overriding capacity, at high concentrations

of glucocorticoids suggests the presence of an 'escape' mechanism to protect the host against overwhelming inflammatory reactions associated with life-threatening trauma or infection.

Monocytes/macrophages are an important source of the MIF that is released by pro-inflammatory stimuli such as LPS and TNF- α (ref. 6). The addition of anti-MIF antibodies to LPS-treated human monocyte cultures was found to potentiate markedly the inhibitory effect of dexamethasone. When human monocytes were stimulated with LPS in the presence of dexamethasone (2×10^{-10} M) and control IgG, TNF- α production was inhibited by only 9.4%, compared with 32.7% for monocytes stimulated with LPS in the presence of dexamethasone (2×10^{-10} M) and neutralizing anti-MIF IgG (Fig. 3). Thus endogenous MIF can act in an autocrine fashion to overcome glucocorticoid inhibition of cytokine production. In an inflammatory site, the production of macrophage-derived cytokines such as TNF- α is likely to reflect the net result of glucocorticoid inhibition and MIF 'overriding'.

Endotoxaemia is associated with a prominent glucocorticoid stress response which, if impaired either by hypophysectomy or adrenalectomy, can lead to fulminant shock and death^{14–17}. The lethal effects of endotoxin are largely due to cytokine activation^{5,18–20}, and several experimental studies have shown that glucocorticoids, when administered early, can be efficacious both in suppressing cytokine production and in promoting survival^{21–23}. We therefore studied the effect of MIF on protection conferred by dexamethasone in an experimental model of endotoxic shock. The administration of rMIF together with

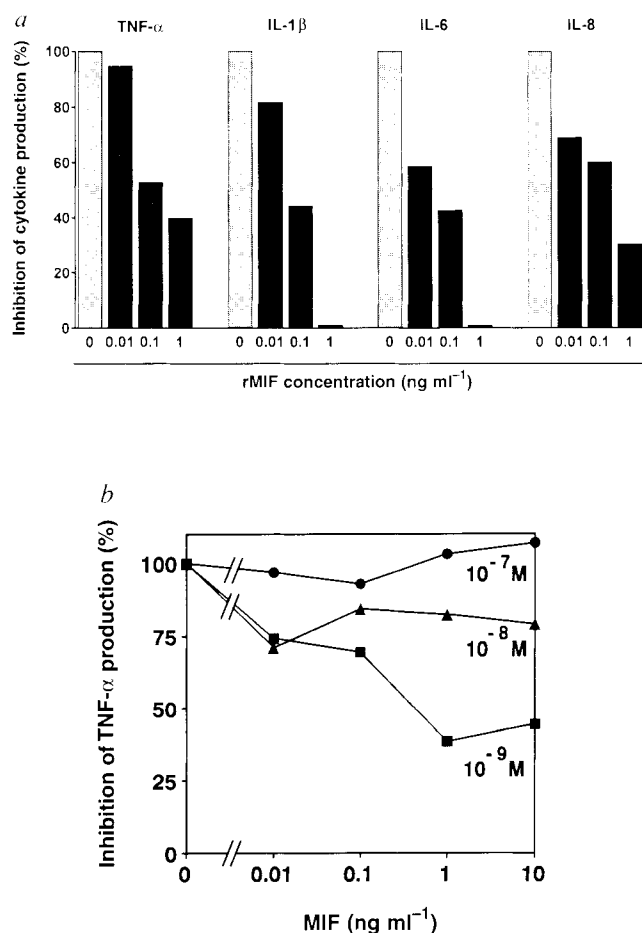


FIG. 3 MIF overrides glucocorticoid-mediated inhibition of cytokine production by LPS-stimulated monocytes. **a**, Human mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation and monocytes purified by adherence. Non-adherent cells were removed by washing each well twice in 24 h with RPMI/10% heat-inactivated FBS. Cells (2×10^6) were preincubated for 1 h with 10^{-9} M dexamethasone or with dexamethasone plus human rMIF (0.01 – 1 ng ml⁻¹ or 0.8 – 80×10^{-12} M) before the addition of $1 \mu\text{g ml}^{-1}$ LPS (*Escherichia coli* O111:B4, Sigma). Human rMIF was prepared as described previously²⁵. Cell culture supernatants were collected after 12 h of stimulation and secreted cytokines quantified by L929 cell cytotoxicity (TNF- α) or by ELISA (IL-1 β , IL-6 and IL-8) (Quantikine; R&D, Minneapolis, MN). MIF has been shown previously not to interfere with the L929 bioassay for TNF- α (ref. 6). Results are expressed as the relative percentage inhibition of cytokine production calculated by the following formula: % inhibition = [(cytokine concentration in culture medium of LPS-stimulated cells) – (cytokine concentration in culture medium of cells treated with dexamethasone plus LPS)] / [(cytokine concentration in culture medium of LPS-stimulated cells) – (cytokine concentration in culture medium of cells treated with dexamethasone plus LPS)] $\times 100$. Data are the mean values of two separate experiments that gave similar results with cells from two different human donors. The MIF effect could not be accounted for by the trace LPS contamination present in the purified, rMIF preparation (≤ 0.5 pg LPS per μg protein). **b**, Effect of dexamethasone concentration on the overriding activity of rMIF. Human monocytes were cultured with LPS and the indicated concentrations of dexamethasone plus rMIF exactly as described above and the conditioned supernatants analysed as in **a**. The concentration of TNF- α was quantified by L929 cell cytotoxicity and the results expressed as the percentage inhibition of cytokine production. Data are the mean values of three separate experiments with cells from three different human donors. For analysing the potentiating activity of anti-MIF antibody on glucocorticoid-inhibition of TNF- α production, 1×10^6 human peripheral blood monocytes were preincubated for 1 h with dexamethasone (1×10^{-10} to 1×10^{-9} M) plus either anti-MIF IgG or control rabbit IgG (each at $25 \mu\text{g ml}^{-1}$) before the addition of LPS ($1 \mu\text{g ml}^{-1}$). Cell culture supernatants were collected after 12 h of stimulation and the concentration of TNF- α was quantified by L929 cell cytotoxicity. The percentage inhibition of TNF- α production was calculated as follows: % inhibition = $100 - (\text{TNF-}\alpha \text{ concentration in culture medium of cells treated with dexamethasone plus antibody plus LPS}) / (\text{TNF-}\alpha \text{ concentration in culture medium of cells treated with LPS})$. Data are the mean of two separate experiments.

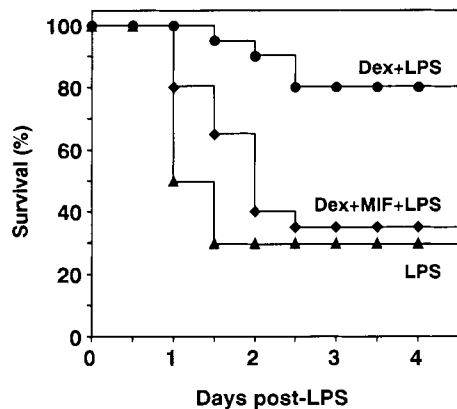


FIG. 4 MIF overrides glucocorticoid inhibition of LPS lethality. Ten-week-old BALB/c mice (19–20 g) were injected intraperitoneally with dexamethasone (Dex) (1.25 mg per kg, that is 25 µg or 67 nmol) with or without murine rMIF (0.6 mg per kg, that is 12 µg or 1 nmol) or saline. After 2 h, all mice were injected intraperitoneally with LPS at a dose of 22.5 mg per kg. MIF-treated mice received an additional MIF injection (0.6 mg per kg) at the time of LPS challenge and 17 h thereafter. The intraperitoneal injection of rMIF at a dose of 1.8 mg per kg produced peak serum MIF levels of 81.7 ± 18.2 ng ml⁻¹. rMIF itself does not exhibit toxicity in this protocol. Data points are from two independent experiments, each of which included 10 mice (Dex + LPS, MIF + Dex + LPS) or 5 mice (LPS) per treatment group ($P = 0.005$ for overall comparison between treatment groups; $P = 0.009$ and $P = 0.01$ for Dex + LPS versus Dex + MIF + LPS or LPS, respectively).

dexamethasone to LPS-treated mice completely blocked the protective effect of dexamethasone on LPS lethality (Fig. 4). Survival rates were 80% in mice treated with dexamethasone + LPS compared with only 35% in those treated with MIF + dexamethasone + LPS, and 30% in mice injected with LPS without dexamethasone. These results provide *in vivo* confirmation of the observation that MIF overcomes the anti-inflammatory effect of glucocorticoids on LPS-induced cytokine production.

Although cytokine mediators such as macrophage colony-stimulating factor (M-CSF), interferon (IFN)-γ or IL-4 may antagonize certain glucocorticoid-mediated effects, MIF is unique in being released from cells after glucocorticoid stimulation. That stress or pro-inflammatory stimulation leads to the production of both anti-inflammatory (glucocorticoid) and pro-inflammatory (MIF) mediators may on first examination appear paradoxical. Yet, in common with many other physiological systems, it is apparent that counter-regulatory mediators are necessary to balance the profound, inhibitory effect of glucocorticoids on the immune system. Within the actual site of an inflammatory reaction, locally produced macrophage- or T-lymphocyte-derived MIF may act to counter-regulate the inhibitory effects of glucocorticoids produced as part of the host stress response. The release of pituitary MIF, however, suggests that the host also has the capacity to counter-regulate the more global anti-inflammatory properties of glucocorticoids that may occur at the circulating, systemic level⁵. We conclude that MIF is a critical component of the immune system and acts together with glucocorticoids to regulate inflammation and immunity. Studies are in progress to determine the molecular basis of the glucocorticoid–MIF interaction at the subcellular level. □

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ACKNOWLEDGEMENTS. We thank K. Manogue, K. Warren and L. Bonetta for reviewing the manuscript, and J. Giorgi and T. D'Amico for technical assistance. This work was supported by the NIH, a grant from the Fonds de perfectionnement du Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland) and the Swiss Foundation for Research in Medicine and Biology.

Therapeutic potentiation of the immune system by costimulatory Schiff-base-forming drugs

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IMMUNE responses are orchestrated by CD4 T lymphocytes, which receive a cognitive signal when clonally distributed receptors are occupied by major histocompatibility complex (MHC) class II-bound peptides on antigen-presenting cells (APCs)^{1,2}. The APCs provide costimulatory signals, through macromolecules such as CD80, that regulate outcomes in terms of T-cell activation or anergy^{3–6}. We have studied essential complementary chemical events in the form of Schiff base formation between carbonyls and amines that are constitutively expressed on presenting cell and T-cell surfaces^{7–9} and provide a new target for manipulation of immune responses^{10,11}. Here we show that small Schiff base-forming molecules can substitute for the physiological donor of carbonyl groups and provide a costimulatory signal to CD4 Th-cells through a mechanism that activates clofilium-sensitive K⁺ and Na⁺ transport. One such molecule, tucareol, enhances CD4 Th-cell responses, selectively favouring a Th1-type profile of cytokine production. *In vivo* tucareol potentially enhances CD4 Th-cell priming and CD8 cytotoxic T-cell priming to viral antigens, and has substantial therapeutic activity in murine models of disease.

We first studied the potent immunopotentiatory properties of small Schiff base-forming molecules as a class in human CD4 T cells responding to antigen *in vitro* (Fig. 1a). Specific murine Th-cell priming *in vivo* is also substantially enhanced after local administration of this class of drug (Fig. 1b). As well as enhancing normal responses mediated by T-cell antigen receptors (TCRs), costimulation by the Schiff base-forming drug tucareol can largely reverse the inhibition that occurs when macromolecular costimulatory pathways are blocked *in vitro* by soluble

Received 31 March; accepted 21 July 1995.

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CTLA-4-Ig fusion protein or by monoclonal antibody to B7-1 (Fig. 1c). In choosing the substituted benzaldehyde tucaresol (4(2-formyl-3-hydroxy-phenoxy)methyl) benzoic acid) for development as an immunopotentiatory drug, we took advantage of an earlier drug development programme aimed at modifying sickle-cell haemoglobin with Schiff base-forming aldehydes¹². From this we knew that certain Schiff base-forming aldehydes had excellent oral bioavailability, pharmacokinetic, and drug safety characteristics, and induced clinical signs, at low doses, consistent with systemic immune hyperreactivity.

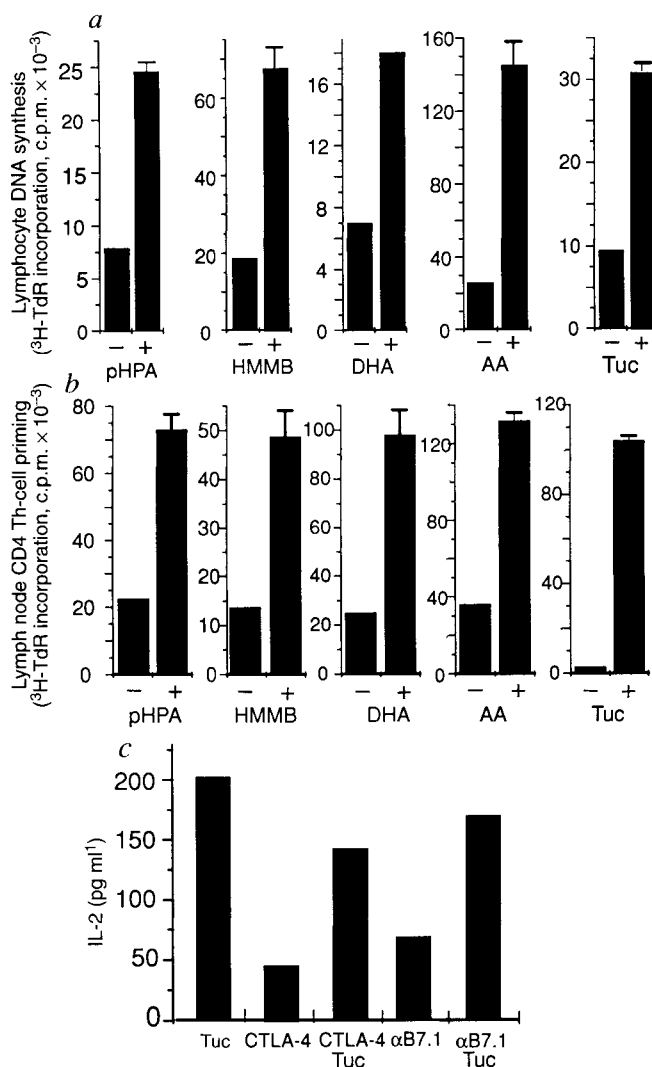
Analysis of tucaresol's costimulatory mechanism of action shows that the drug forms a Schiff base on CD4⁺ T-cell-surface amines that can be visualized by competitive covalent ligation with sulphonated *N*-hydroxysuccinimidobiotin (S-NHS-biotin) and flow cytofluorimetry (Fig. 2a). Prior ligation of cell-surface amines by S-NHS-biotin prevents the immunopotentiatory effect of tucaresol (Fig. 2b), but does not affect responses to stimuli that are independent of costimulatory signals, such as phorbol myristate acetate/Ca²⁺ ionophore (data not shown). Schiff base formation by tucaresol substantially enhances interleukin (IL)-2 production in response to CD3 ligation, with a characteristic bell-shaped dose-response relation (Fig. 2c). In contrast, the production of IL-4 in response to CD3 ligation is reduced in the presence of tucaresol (Fig. 2d). Release of interferon (IFN)- γ in response to TCR-CD3 signalling is also substantially enhanced by tucaresol, whereas IL-6 is unaffected by immunopotentiatory concentrations of the drug (Fig. 2e, f), indicating that

tucaresol strongly favours a Th1-type profile of cytokine production. The highly consistent bell-shaped immunopotentiatory dose-response curve (which occurs both *in vitro* and *in vivo*) accords with expectation if physiological Schiff base formation is important in APC:T-cell adhesion as well as signalling. In this case, increasing concentrations of tucaresol will begin to interfere with APC:T-cell conjugation as the availability of functionally important cell-surface amines becomes limiting.

Electron probe X-ray microanalysis^{13,14} of tucaresol-treated cells shows that the primary consequence of Schiff base formation on CD4⁺ T-cell-surface amines is a pronounced fall in intracellular potassium and a rise in intracellular sodium, fully evident within 5 minutes and returning to the resting state within 2 hours (Fig. 3a). These effects, like the enhancement of IL-2 production by tucaresol, are completely prevented by prior covalent ligation of cell-surface amines with S-NHS biotin (Fig. 3b). Importantly, both the tucaresol-induced changes in intracellular potassium and sodium, and tucaresol-induced enhancement of IL-2 production in response to TCR-CD3 signalling, are completely inhibited by the selective K⁺ channel antagonist clofilium tosylate (Fig. 3c, d). Tucaresol has no effect on the increase in intracellular free Ca²⁺ induced by TCR-CD3 signalling (data not shown).

In vivo, tucaresol forms readily reversible Schiff base adducts with plasma proteins, haemoglobin and other functionally inert amino groups to create a persistent source of free drug, providing costimulation to T cells in the presence of antigen. Enhancement of CD4 Th-cell priming to viral antigens by systemic tucaresol

FIG. 1 a, Human peripheral blood Th-cell proliferative responses to antigen (Ag) *in vitro* in the absence (–) or presence (+) of Schiff base-forming aldehydes and ketones. pHPA, 175 μ M p-hydroxyphenylacetaldehyde (tetanus toxoid as Ag); HMMB, 10 μ M 3-hydroxy-1-(4-methoxyphenyl)-3-methyl-2-butanone (monocyte alloantigens as Ag); DHA, 700 μ M dioxoheptanoic acid (tetanus toxoid as Ag); AA, 1 mM acetaldehyde (tetanus toxoid as Ag); Tuc, 500 μ M tucaresol (4(2-formyl-3-hydroxy-phenoxy)methyl) benzoic acid) (tetanus toxoid as Ag). The relative potency of compounds is independent of the composition of Ag and is similar when compared using the same Ag (data not shown). Assays were performed as previously described⁷. Compounds were added 24 h before Ag. b, Murine CD4 Th-cell priming *in vivo* in the absence (–) or presence (+) of Schiff base-forming aldehydes administered subcutaneously at the site of immunization. pHPA, 3 once-daily doses of 1 mg pHPA with 1 μ g r-nucleoprotein of A/PR8 influenza (NP) in B10S mice; HMMB, 4 once-daily 1 mg doses with 10 μ g keyhole limpet haemocyanin (KLH) in Balb/c mice; DHA, 4 once-daily 1 mg doses with 10 μ g KLH in Balb/c mice; AA, single 1 mg dose with 50 μ g KLH in Balb/c mice; Tuc, single 1 mg dose with 10 μ g NP peptide 265–283 in B10S mice. Mice were immunized subcutaneously in the dorsal midline at the tailbase. The first (or single) dose of compound was mixed with Ag in aqueous solution. Regional (inguinal) lymph nodes were removed after 7 days and restimulated with antigen as previously described¹⁰. c, TCR-mediated responses inhibited by soluble CTLA-4-Ig or anti-B7-1 *in vitro* are restored to near normal levels by 300 μ M tucaresol. Human peripheral blood mononuclear cells were stimulated with 250 ng ml^{–1} anti-CD3 (clone UCH-T1, Sera Lab, Sussex). Cells were exposed to 10 μ g ml^{–1} CTLA-4-Ig (a fusion protein)²⁵ for 30 min at 4 °C before stimulation. After 24 h, supernatants were assayed for IL-2 content by ELISA using a commercial kit (Quantikine immunoassay kit, R&D Systems, Minneapolis, MN). The same protocol was used for inhibition by monoclonal antibody to B7-1 (CD80) at 20 μ g ml^{–1} (clone BB-1, Serotec, Kidlington, Oxfordshire).



is shown in Fig. 4a. However, this agent is also potentially exploitable as a vaccine adjuvant. Local tucaresol is more effective than conventional adjuvants in priming Th cells with a peptide vaccine, and combination with conventional adjuvants, used with rHBV core protein, indicates that tucaresol is compatible with alum but not with incomplete Freund's adjuvant (Fig. 4b). The mechanisms determining compatibility are presently unknown, but conceivably an oil emulsion could be unfavourable for delivery of the water-soluble drug to cell-surface amines.

Enhancement of CD8 cytotoxic T-cell priming to influenza peptide antigens by systemic tucaresol (administered parenterally) is shown in Fig. 4c, d. At immunopotentiatory doses, systemic tucaresol is therapeutically effective in a murine model of cytomegalovirus infection¹⁵ (Fig. 4e) and in a model of syngeneic tumour growth using the murine colon adenocarcinoma MCA38 (Fig. 4f). Immunopotentiation and therapeutic efficacy invariably exhibit a bell-shaped curve.

Further work will seek to identify the complete pathway of costimulation by tucaresol. Clofilium-sensitive K^+ channels have already been implicated in the activation pathway leading to IL-2 production¹⁶, but other classes of K^+ - and Na^+ -permeant proteins could also be involved¹⁷. Convergence of Schiff base signalling with TCR signalling has been identified at the level of

tyrosyl phosphorylation of the MAP-kinase ERK-2 (Chen, H., Rogers, M. & Rhodes, J., manuscript in preparation), an intermediary element in the TCR-CD3 signalling pathway¹⁸. A distinct therapeutic advantage of small Schiff base-forming drugs over cytokine therapy is likely to be the avoidance of toxicity through cytokine cascade effects. Promotion of a Th1-like profile of cytokine production and selective enhancement of cell-mediated responses is likely to be therapeutically favourable against intracellular pathogens such as viruses, mycobacteria and protozoal parasites^{19,20}. In HIV infection the situation is less clear^{21,23}, but enhancement of CTL responses is likely to be therapeutically desirable²⁴. In a small study of four macaques infected with simian immunodeficiency virus, tucaresol produced no exacerbation of infection (Stott, E. J., Chan, L., Kent, K. & Almond, N., unpublished observation), rather a >3 log and 1 log reduction in viral loads were noted in the two treated animals, the latter being within the limits of normal variability. Despite increasing knowledge of immune mechanisms at the molecular level, no rationally based immunopotentiatory drugs have been available for clinical use. Our results establish the importance of Schiff base formation in the induction of immune responses and provide a new costimulatory mechanism in CD4 Th cells. They also allow the rational development of orally bioavailable immuno-

FIG. 2 a, Schiff base formation between tucaresol and cell-surface amines on CD4⁺ T cells demonstrated by competitive covalent ligation with S-NHS-biotin and FACS analysis. Untreated cells stained with S-NHS biotin (I); cells stained with S-NHS-biotin pretreated with 300 μ M tucaresol (II), 600 μ M tucaresol (III), 1,200 μ M tucaresol (IV) and 2,500 μ M tucaresol (V). CD4⁺ cells were isolated by means of IsoCell CD4 isolation kits (Pearce & Warriner, Cheshire). Cells in PBS were incubated for 30 min at 37 °C with tucaresol. The selective reducing agent NaCNBH₃ (10 mM) (Sigma) at pH 7.4 was then added. After 1 h at 20 °C, cells were washed 3 times and exposed to 12.5 μ M S-NHS-biotin (Pierce Rockford, IL) for 1 h at 37 °C to ligate free surface amino groups. After a further 2 washes, cells were exposed to fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, Peterborough) (1:50 dilution) for 30 min at 4 °C. Cells were fixed with 1% paraformaldehyde and subjected to FACS analysis as previously described¹⁰. b, Enhancement of IL-2 production in response to TCR-CD3 signalling by tucaresol is inhibited by prior ligation of cell-surface amines by S-NHS biotin. Inhibition is not complete because some regeneration of free amino groups occurs over 24 h. S-NHS-biotin treatment (100 μ M) was as described above. 300 μ M tucaresol was added to S-NHS biotin treated and untreated PBMC cultures at time 0. After 24 h, 125 μ g ml⁻¹ anti-CD3 (Clone UCH-T1, Sera Lab, Sussex) was added. Cell-free supernatants were collected 24 h later and assayed for IL-2 content. In the following studies, tucaresol was added to PBMC cultures at time 0 and anti-CD3 24 h later. Supernatants were collected after 48 h and assayed for cytokine content using commercial ELISA kits. c, Enhancement of CD3-TCR-induced IL-2 production by tucaresol exhibits a bell-shaped curve. Anti-CD3 at 250 ng ml⁻¹ (squares), 125 ng ml⁻¹ (circles), 62.5 ng ml⁻¹ (triangles), and no anti-CD3 (inverted triangles). d, IL-4 production in response to anti-CD3 is reduced in the presence of tucaresol. Anti-CD3 at 250 ng ml⁻¹ (squares), 125 ng ml⁻¹ (circles), 62.5 ng ml⁻¹ (triangles), and no anti-CD3 (inverted triangles). e, Tucaresol enhances the production of IFN- γ in response to anti-CD3 signalling, and exhibits a bell-shaped dose-response relation. Anti-CD3 at 250 ng ml⁻¹ (squares), 125 ng ml⁻¹ (circles), 62.5 ng ml⁻¹ (triangles), and no anti-CD3 (inverted triangles). f, Tucaresol has no effect on IL-6 production in PBMC cultures stimulated with anti-CD3. Anti-CD3 at 250 ng ml⁻¹ (triangles), 125 ng ml⁻¹ (circles), and no anti-CD3 (squares).

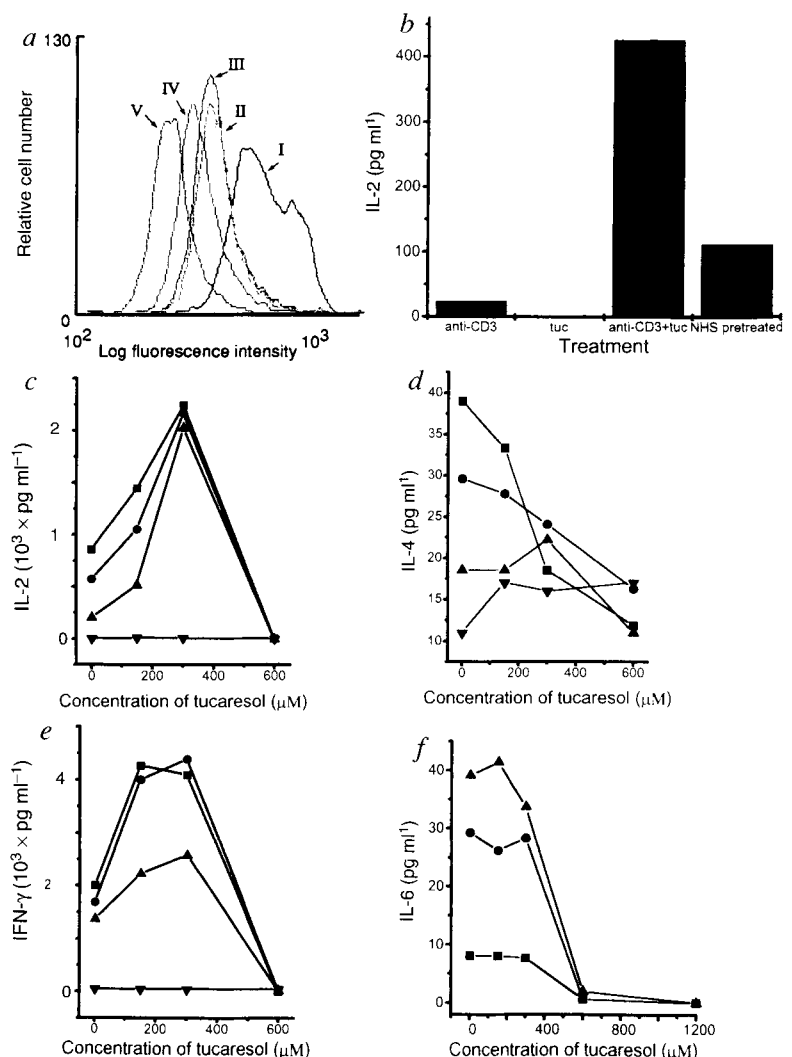


FIG. 3 a, Electron-probe X-ray microanalysis of elemental Na, K and Cl in peripheral blood lymphocytes treated with tucareosol. Tucareosol (300 μ M) induces a pronounced fall in potassium and a concomitant rise in sodium, fully evident within 5 min and returning to the resting state by 2 h. Elemental concentrations are expressed as ratios with intracellular phosphorus (which remains constant). Identical results were obtained in purified CD4⁺ cells. **b**, The activation of K and Na transport depends on the formation of Schiff bases between tucareosol and cell-surface amines and is completely prevented by prior ligation of these groups in an amide linkage with S-NHS-biotin. Pretreatment with S-NHS-biotin was as described above. K and Na concentrations were measured at the 10-min time point. **c**, Tucareosol's effects are inhibited by the K⁺ channel antagonist clofilium tosylate (30 μ M) added 30 s before tucareosol. Elemental concentrations were measured at the 10-min time point. **d**, Enhancement of IL-2 production in response to TCR-CD3 signalling by tucareosol is completely inhibited by clofilium added simultaneously with tucareosol (squares). In marked contrast, no inhibition is observed when the addition of clofilium is delayed for 24 h (circles) (until the time of anti-CD3 stimulation), to allow time for the delivery of the costimulatory signal by tucareosol. Inhibition by clofilium is complete (is not confined to reversal of the enhancement produced by tucareosol), and this is expected if tucareosol is amplifying an essential costimulatory signal provided by Schiff base formation between constitutive cellular ligands.

METHODS. Three prewarmed replicates of tucareosol-treated or PBS-treated cells in RPMI 1640 containing 10% autologous serum were incubated for specified periods at 37 °C in an atmosphere of 5% CO₂ in air. Cell suspensions were dispensed onto a 25-mm nucleopore filter (3 μ m pore size) (Costar), washed with 1 ml of 0.3 M sucrose and attached to the filter by vacuum filtration. Cells on filters were slam-frozen against a liquid nitrogen-cooled copper mirror using an MM80 (Leica) and freeze-dried overnight. Preparations were mounted on aluminium stubs using conductive carbon cement (Neubauer), carbon-coated and analysed using an EDAX PV9900 analyser with a Philips PSEM 500 at 12 kV. Count rate was stabilized at 1,000 c.p.s. and spectra acquired for a live time of 100 s. Ten spectra were collected for each of the 3 replicates. Peak-to-background ratios of the elements of interest were normalized to phosphorus for each cell and means of the replicate groups compared using a one-way ANOVA.

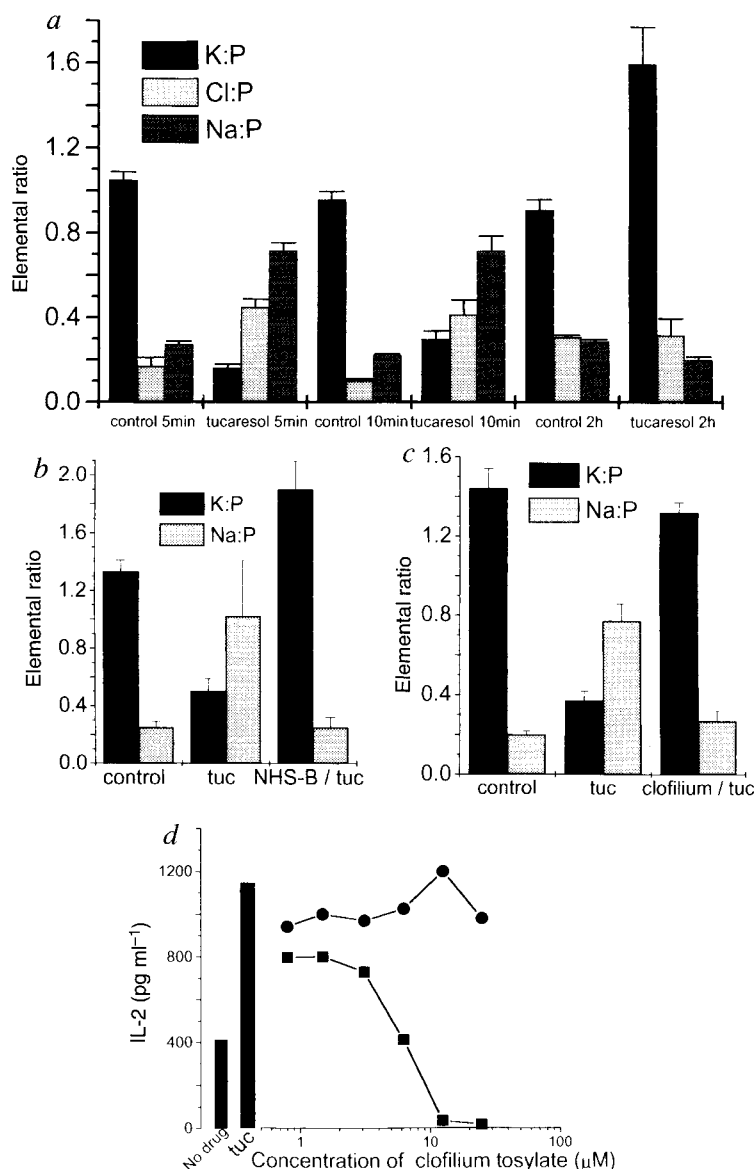


FIG. 4 a, Systemic enhancement of murine CD4 Th-cell priming to human r-hepatitis B virus core protein (r-HBc) by parenteral and oral tucareosol. Left: no immunization (squares), immunized, no treatment (circles), 20 mg per kg tucareosol (triangles). Right: no immunization (squares), immunized, no treatment (circles), 5 mg per kg oral tucareosol (triangles); 10 mg per kg tucareosol (inverted triangles); 20 mg per kg tucareosol (diamonds); 50 mg per kg tucareosol (no symbols). Balb/c mice were immunized with r-HBc (500 ng) subcutaneously at tailbase. Tucareosol was administered once daily for 5 days beginning on the day of immunization. On day 7, regional (inguinal) lymph nodes were removed and restimulated with r-HBc. **b**, Adjuvant effects of tucareosol. B10S mice were immunized with 10 μ g NP peptide 265–283 (B10S Th-cell epitope¹⁰). The standard dose of adjuvant¹⁰ or 1 mg of tucareosol mixed with Ag was administered. After 7 days, lymph node cells were restimulated with Ag *in vitro*. Columns show (left to right) alum (Wellcome) tucareosol, saponin (Quil A Superfos, Denmark) and tucareosol. Balb/c mice were immunized with 2 μ g rHBc. Columns show (left to right) incomplete Freund's adjuvant (IFA), IFA plus tucareosol (1 mg), tucareosol (1 mg) alone, alum, alum plus tucareosol (1 mg), and tucareosol (1 mg) alone. **c**, Enhancement of CD8 cytotoxic T-cell priming to influenza peptides by systemic tucareosol (peptide-pulsed target cells). Live virus infection (open squares), Freund's complete adjuvant (FCA) (filled squares), local + systemic tucareosol (filled diamonds), systemic tucareosol (filled circles), peptide only (no adjuvant or drug) (open triangles), local + systemic tucareosol, Ag-negative targets (inverted filled triangles), systemic tucareosol, Ag-negative targets (filled triangles). **d**, Enhance-

ment of CD8 cytotoxic T-cell priming to influenza peptides by tucareosol (virus infected target cells). FCA (filled squares), local + systemic tucareosol (filled diamonds), systemic tucareosol (filled circles), peptide only (open squares), local + systemic tucareosol, Ag-negative targets (inverted filled triangles), systemic tucareosol, Ag-negative targets (inverted open triangles). Balb/c mice were immunized with 100 μ g of a hybrid peptide containing CTL (147–160) and Th-cell (216–229) epitopes of A/PR8 NP, and boosted 3 weeks later with 10 μ g r-NP plus 100 μ g of peptide intraperitoneally (i.p.). Five groups (four per group) received the following: 1, no adjuvant or drug; 2, primary subcutaneous immunization in FCA and boosting in IFA; 3, tucareosol (200 μ g) with the priming Ag followed by tucareosol i.p. (200 μ g) once daily for 4 days. At boosting, 200 μ g tucareosol i.p., repeated for the next 3 days; 4, tucareosol i.p. (200 μ g) on day of priming and daily for the next 4 days. At boosting, 200 μ g tucareosol i.p., repeated for the next 3 days; 5, live virus (20 haemagglutinating units) administered intravenously (no immunization). Two weeks after boosting, spleens were removed and spleen cells restimulated *in vitro* with peptide (30 μ g ml⁻¹) for 5 days. Cytotoxicity assays were performed on peptide-pulsed or virus infected ⁵¹chromium-labelled P815-J cells as previously described¹⁰. **e**, Efficacy of systemic tucareosol in a murine model of cytomegalovirus infection. Virus recovered from untreated mice (–), mice treated with 10 mg (+) and 50 mg per kg tucareosol (++) Balb/c mice were infected i.p. with 10⁴ plaque forming units (PFU) of murine cytomegalovirus (MCMV) Osborn. Mice received tucareosol i.p. once daily for 6 days beginning 1 day before infection. 7 days after infection, splenic viral load was measured as

potentiatory drugs with broad potential in the treatment of chronic infectious disease and adjunct therapy for cancer. □

Received 4 April; accepted 20 July 1995.

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ACKNOWLEDGEMENTS. We thank B. Pearce, L. Harryman, D. Gower and M. Trowbridge for assistance, and D. Rowlands for gifts of recombinant Hbc. CTLA-4-Ig was generously provided by S. Gorman and S. Crowe.

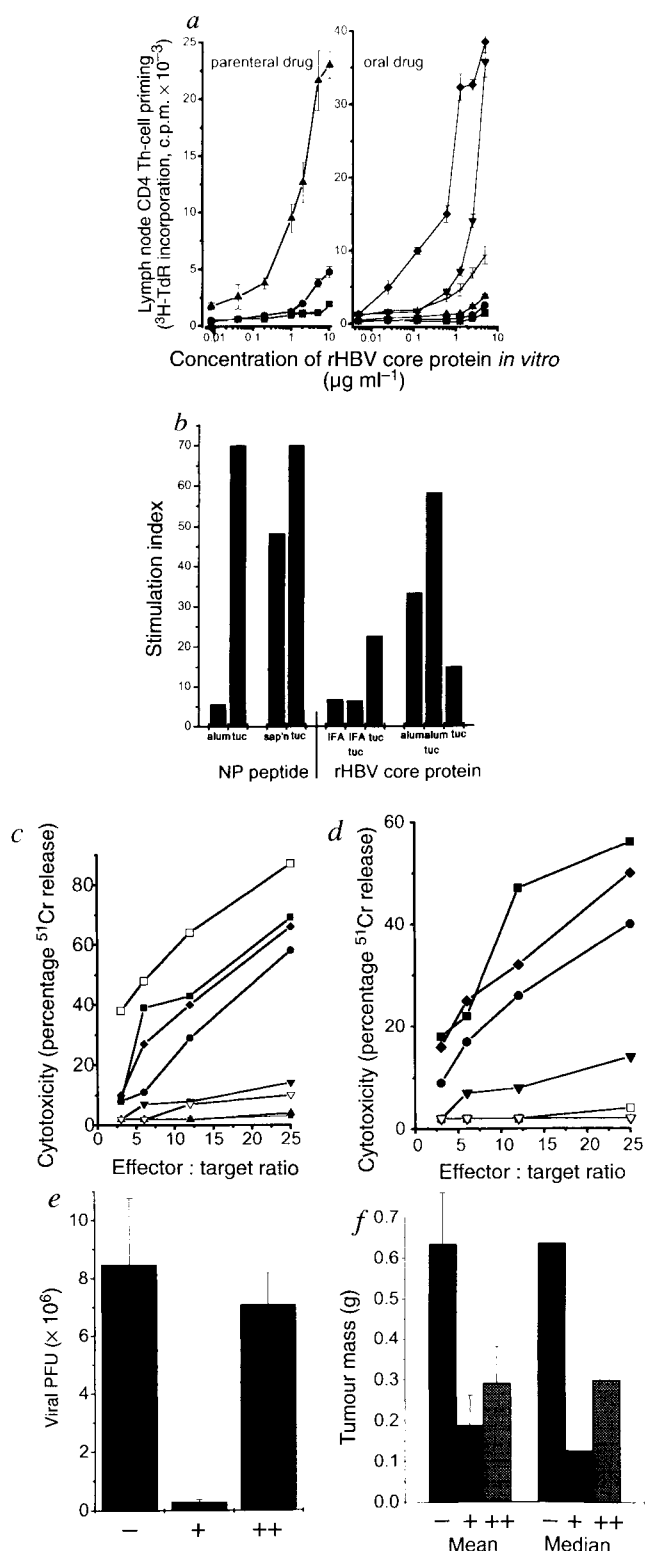
Ca²⁺- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils

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CHEMOATTRACTANTS stimulate neutrophil migration by activating signalling pathways¹ including repeated transient increases in intracellular free calcium, [Ca²⁺]_i (refs 2, 3). A motile neutrophil sends out many pseudopods, some of which adhere to the substrate; to continue moving forward the cell must release these attachments^{4,5}. Adhesion can be actively regulated, and neutrophils in which [Ca²⁺]_i transients are inhibited become stuck on fibronectin or vitronectin^{6,7}, extracellular matrix proteins that neutrophils encounter *in vivo*. Function-blocking antibodies to β3 integrins or the αvβ3 heterodimer restore motility on vitronectin to [Ca²⁺]_i-buffered cells (B. Hendey, M.A.L., E. Marcantonio and F.R.M., manuscript submitted), indicating that an αvβ3-like integrin is responsible for the [Ca²⁺]_i-sensitive adhesion. We show that the density of αvβ3 integrins in the adherent membrane of neutrophils migrating on vitronectin is much higher at the leading edge than at the rear, but [Ca²⁺]_i buffering or inhibition of Ca²⁺-calmodulin-activated protein phosphatase 2B (calcineurin) leads to accumulation of αvβ3 on the adherent surface at the rear of the cell. We show that the polarized distribution of αvβ3 integrins in migrating neutrophils is maintained by [Ca²⁺]_i-dependent release of adhesion followed by endocytosis of these integrins and recycling to the leading edge.

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PFU per g by plaque assay. Tucarecol has no direct anti-CMV effects *in vitro*. **f**, Efficacy of systemic tucarecol in a model of syngeneic tumour growth. Mean and median tumour mass in untreated mice (-), mice treated with 10 mg per kg (+) and 50 mg (++) tucarecol. The weakly immunogenic murine colon adenocarcinoma MCA38 was implanted subcutaneously in syngeneic C57BL/6 mice as 2-mm cubes by trocar and canula in the right flank. Treated groups (10 mice per group) received 9 i.p. injections of tucarecol on alternate days, commencing on day 5 after implantation (to allow tumour establishment). Control mice received PBS. Tumours were excised and weighed on day 24. Tucarecol had no effect on the growth of MCA38 adenocarcinoma in CD1 nu/nu (athymic) mice (data not shown).

To investigate the proteins that are directly involved in neutrophil adhesion to vitronectin, we used a shearing procedure⁸ that leaves behind only the lower adherent membrane (Fig. 1*a, b*). In the adherent membranes of migrating cells, both α v and β 3 integrin chains are found at the leading edge, along with F-actin (Fig. 1*c, d, f, g*). The presence of these integrins on the extending

pseudopod makes them available for adhesion to vitronectin at the front of the migrating cell. In contrast, when $[\text{Ca}^{2+}]_i$ is buffered, β 3 integrins (Fig. 1*e, h*) are concentrated toward the rear of the cells, and the leading edge (which is still rich in F-actin) is nearly devoid of these integrins. α v Integrin chains are also clustered at the rear of $[\text{Ca}^{2+}]_i$ -buffered cells (not shown). This

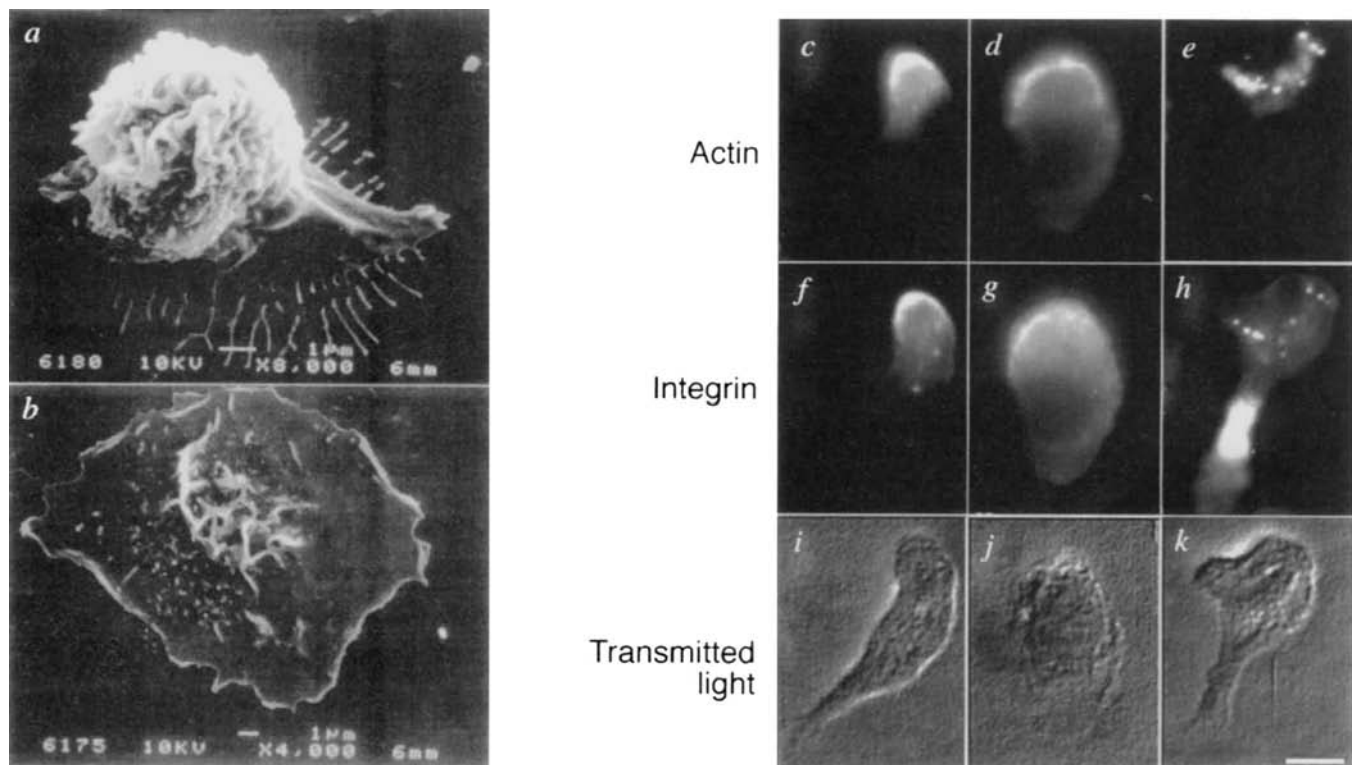


FIG. 1 *a, b*, Scanning electron micrographs of neutrophil adherent membranes before (*a*) and after (*b*) shearing. *c–k*, Effect of $[\text{Ca}^{2+}]_i$ -buffering on integrin localization in neutrophils on vitronectin. Neutrophils on vitronectin were stimulated to migrate with *N*-formyl-Met-Leu-Phe (fMLP) and then ruptured leaving only adherent membranes. Rhodamine-phalloidin staining was used to determine the orientation of cells because F-actin is found predominantly at the leading edge of migrating neutrophils²⁷. By examination of whole cells we have verified that F-actin is strongly enriched at the front of $[\text{Ca}^{2+}]_i$ -buffered neutrophils. This leading-edge localization of F-actin is seen clearly in sheared adherent membranes (*c–e*). Antibodies to the cytoplasmic domains of integrins were used to determine their distribution (*f–h*). Enhanced transmitted-light images of the same cells are shown (*i–k*) to illustrate the shape of the adherent membranes. As when living neutrophils are observed migrating on adherent proteins^{2,6,7,9}, the attached membranes show a variety of shapes from elongated to round. Where indicated, the $[\text{Ca}^{2+}]_i$ was buffered with QUIN-2. In control cells α v and β 3 integrins are predominantly at the leading edge; in $[\text{Ca}^{2+}]_i$ -buffered cells most β 3 integrin is at the rear of the cell. In 5 experiments, 82% ($n=114$) of control cells showed β 3 integrin concentrated heavily at the front of the cell, whereas in 76% ($n=96$) of the $[\text{Ca}^{2+}]_i$ -buffered cells the β 3 integrin was clustered at the rear. Similar percentages were seen for α v integrins in 3 experiments. $[\text{Ca}^{2+}]_i$ transients can also be suppressed within 5 min by incubating neutrophils in medium containing EGTA², and similar effects on α v β 3 localization were obtained under these conditions. Scale bar, 5 μ m.

METHODS. Neutrophils were isolated from whole blood donated by healthy volunteers by a single-step separation over a Ficoll-Hypaque solution (Gibco BRL, Gaithersburg, MD, USA). Contaminating erythrocytes were lysed by a 30-s hypotonic shock. Cells were then rinsed with PBS and resuspended in incubation buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 20 mM HEPES, pH 7.4). Where indicated,

cells were incubated in a solution of 50 μ M of the $[\text{Ca}^{2+}]_i$ buffer QUIN-2/AM (Molecular Probes, Eugene, OR, USA) for 40 min. The $[\text{Ca}^{2+}]_i$ of the buffered cells remains at approximately 50 nM after loading². The cells were plated for 5 min at 37 °C on coverslip dishes that had been previously coated for 1 h with a solution of 10 μ g ml⁻¹ vitronectin (Gibco BRL). Cells were then stimulated for 5 min with 10 nM fMLP. The cells were ruptured with a 2-ml jet of standard breaking solution (SBS)⁸ delivered from a 21-gauge needle. For scanning electron microscopy (*a, b*), cells were fixed for 30 min in 2.5% (v/v) glutaraldehyde and rinsed with PBS. The samples were serially dehydrated in graded ethanol, and critical-point dried in CO_2 in a Tousimis Samdri 790 (Rockville, MD, USA). The samples were then sputter-coated with gold-palladium in a Denton Vacuum Desk 1 (Cherry Hill, NJ, USA) and viewed at 10 kV on a Jeol 6400 scanning electron microscope (Peabody, MA, USA). For fluorescence microscopy (*c–k*), membranes were fixed with 4% paraformaldehyde in PBS for 4 min and incubated for 10 min with PBS containing 10% (w/v) calf serum and 2 mg ml⁻¹ BSA (immunostaining buffer) and 1 μ g ml⁻¹ aprotinin, 1 μ g ml⁻¹ leupeptin and 1 μ g ml⁻¹ anti-pain (Sigma). Samples were stained with 0.3 μ g ml⁻¹ rhodamine phalloidin (Sigma) and 1:100 dilution of a polyclonal antibody to the cytoplasmic domain of α v integrin (Chemicon, Temecula, CA, USA) or 1 μ g ml⁻¹ monoclonal antibody to the cytoplasmic domain of β 3 integrin (Bioreclamation, East Meadow, NY, USA) in immunostaining buffer for 1 h. Samples were rinsed with immunostaining buffer and incubated in a fluorescein-conjugated secondary antibody for an additional 1 h. The samples were rinsed again and visualized as described (B. Hendey, M.A.L., E. Marcantonio and F.R.M., manuscript submitted). Transmitted-light images were obtained with phase-contrast optics and enhanced by convolution with a shadowing filter using NIH Image 1.55 (Wayne Resband, NIH, Bethesda, MD, USA, program available by email from zippy.nimh.nih.gov). This enhancement was necessary because the thin membranes showed weak contrast by conventional microscopy.

localization of $\alpha\beta3$ integrins accounts for many of the motility properties of $[\text{Ca}^{2+}]_i$ -buffered cells on vitronectin. The cells are firmly anchored at the rear and do not move forward, although they continue to put out pseudopods that do not attach to the substrate^{2,7,9}.

The polarized distribution of $\alpha\beta3$ integrins is substrate-dependent. On fibronectin, $\alpha\beta$ integrin is found throughout the cells both in internal vesicles and on the cell surface, and the localization is unaffected by $[\text{Ca}^{2+}]_i$ buffering (not shown). Antibodies to $\alpha\beta3$ integrins do not restore motility to $[\text{Ca}^{2+}]_i$ -buffered cells on fibronectin. These data indicate that $\alpha\beta3$ integrins become polarized in their distribution only when they are on a substrate to which they bind tightly.

The most abundant integrins on neutrophils are members of the $\beta2$ family (CD18)^{10,11}. However, antibodies to $\beta2$ integrins do not restore motility to $[\text{Ca}^{2+}]_i$ -buffered neutrophils on vitronectin (B. Hendey, M.A.L., E. Marcantonio and F.R.M., manuscript submitted). When cells were stained for $\beta2$ integrins, the staining was seen uniformly over the cell surface, and this localization was unaffected by $[\text{Ca}^{2+}]_i$ buffering (results not shown). These data are consistent with release of $\beta2$ integrin attachments independent of $[\text{Ca}^{2+}]_i$ signalling.

Integrins on the adherent surfaces of cells are associated with proteins that link the actin cytoskeleton to integrins, including talin^{12,13}, α -actinin^{14,15} and vinculin¹⁶. In migrating neutrophils, talin is localized with F-actin at the leading edge (Fig. 2a, c, e). However, in $[\text{Ca}^{2+}]_i$ -buffered cells, talin is in clusters toward the rear of the cells and is clearly separated from the F-actin at the leading lamellipod (Fig. 2b, d, f). $\alpha\beta$ Integrin colocalizes with talin and with α -actinin in migrating and in $[\text{Ca}^{2+}]_i$ -buffered cells (not shown). These results suggest that adhesion complexes with $\alpha\beta3$ are formed at the leading edge of migrating neutrophils, and $[\text{Ca}^{2+}]_i$ increases are required to break them apart.

Inhibition of the Ca^{2+} -dependent phosphatase calcineurin causes neutrophils to become stuck on vitronectin in the same way as when $[\text{Ca}^{2+}]_i$ is buffered⁹. A calcineurin inhibitor peptide, CN412, was introduced into cells using a procedure involving endocytosis and osmotic shock⁹. Both $\beta3$ (Fig. 2g–l) and $\alpha\beta$ integrins (not shown) were found in clusters toward the rear of CN412-treated cells, very similar to the localization seen in $[\text{Ca}^{2+}]_i$ -buffered cells. This suggests that dephosphorylation by calcineurin of a protein in the adhesion complexes is required for the breakup of $\alpha\beta3$ clusters and detachment of neutrophils from vitronectin. Although there is evidence that serine/

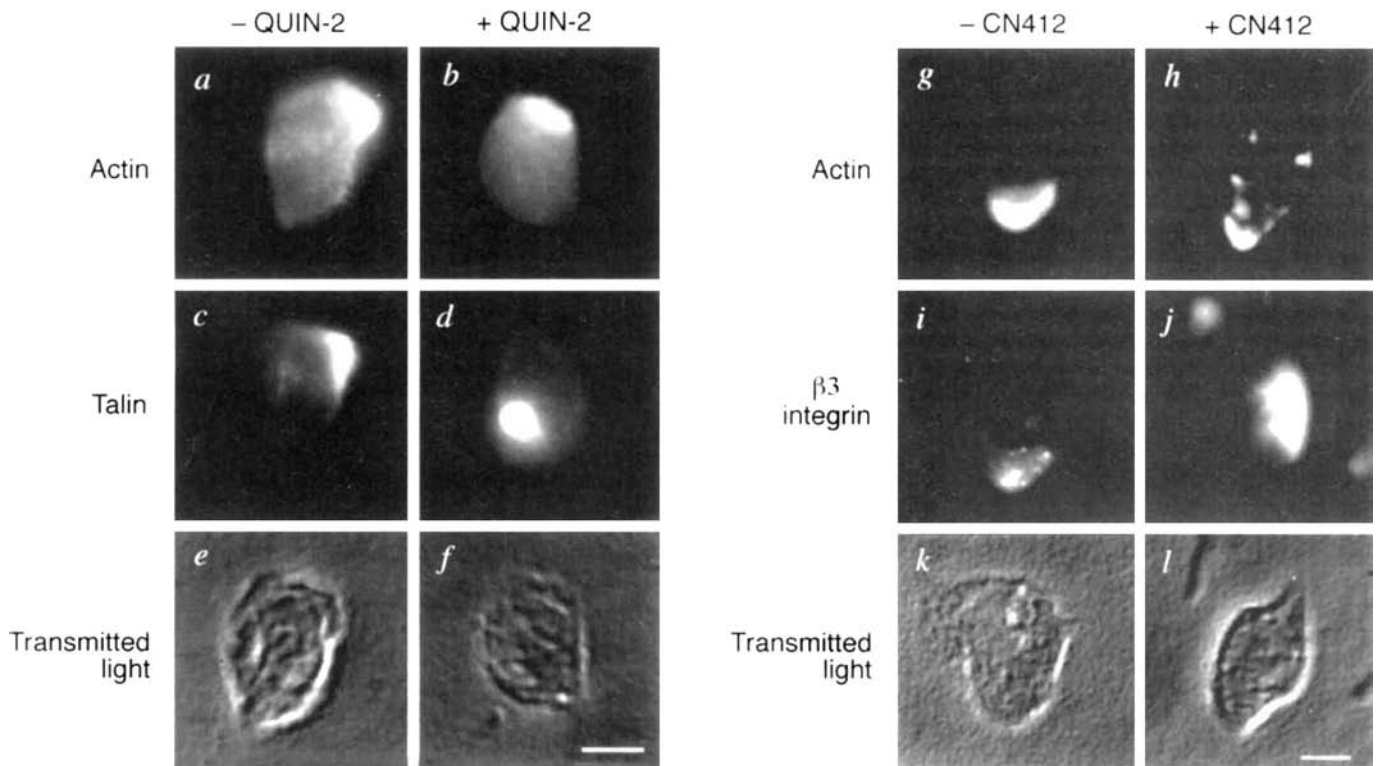
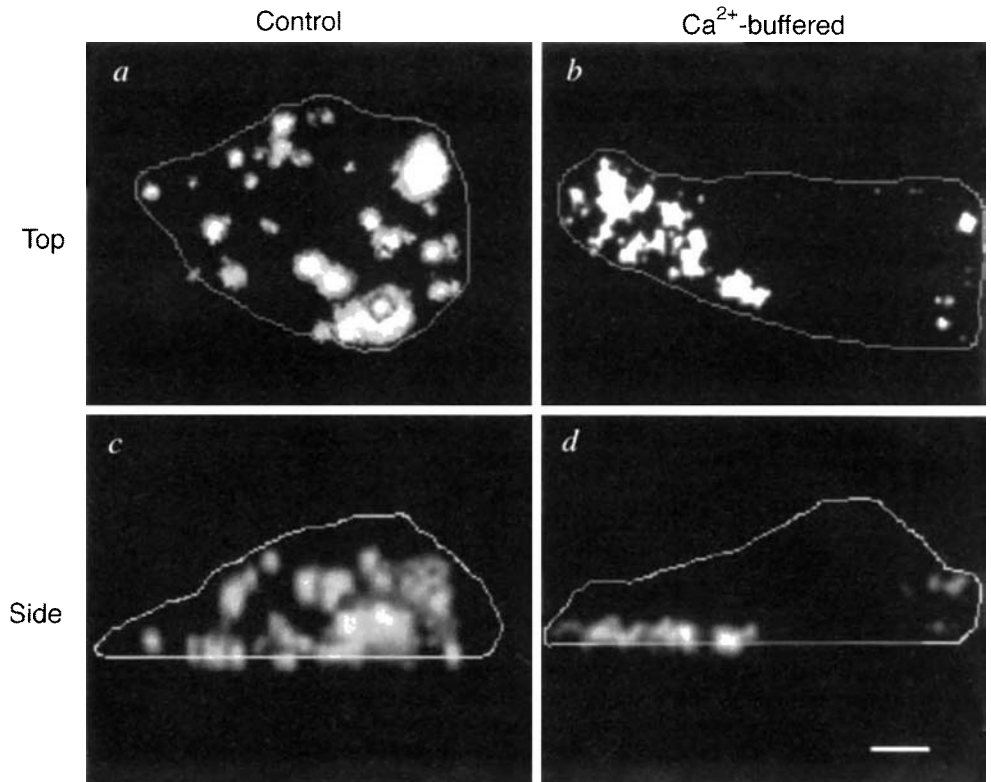


FIG. 2 a–f, The effects of $[\text{Ca}^{2+}]_i$ buffering on talin localization. The leading edge of FMLP-stimulated neutrophils was localized with phalloidin staining of F-actin (a, b). Talin is at the leading edge of control cells (c) but in clusters at the rear of $[\text{Ca}^{2+}]_i$ -buffered cells (d). More than 75 cells in 3 experiments were examined: 84% ($n=99$) of control cells showed colocalization of talin with phalloidin at the leading edge, and 77% ($n=85$) of $[\text{Ca}^{2+}]_i$ -buffered cells showed clusters of talin at the rear. Enhanced transmitted light images are shown (e, f). g–l, Effect of calcineurin inhibition on integrin clusters. In control cells, the $\beta3$ integrin (i) colocalizes with F-actin (g). After calcineurin inhibition with CN412 peptide, the $\beta3$ integrin is localized in clusters at the rear of the cell (j) well away from the leading edge (h). Enhanced transmitted-light images of adherent membranes are shown (k, l). In 3 experiments ($n=81$) 82% of control cells showed $\beta3$ integrin at leading edge, and 86% of CN412-loaded cells ($n=84$) showed $\beta3$ integrin at the rear of the cells. Scale bar, 5 μm .

METHODS. Adherent membranes were prepared and fixed as described for Fig. 1. F-actin was localized with rhodamine-labelled phalloidin as for Fig. 1. Talin was localized with a monoclonal antibody ($1 \mu\text{g ml}^{-1}$) to talin (Chemicon) and a fluorescein-labelled secondary antibody. Where indicated, cells were incubated with 100 μM CN412 for 30 min at 37 $^{\circ}\text{C}$ to allow endocytosis of the calcineurin inhibitor peptide. Cells were then subjected to osmotic shock in H_2O without added salts for 30 s to disrupt endocytotic vesicles, thereby introducing the peptide into the cytoplasm⁹. Control cells were subjected to osmotic shock in the absence of the peptide. The cells were then rinsed thoroughly with PBS and resuspended in incubation buffer. After the osmotic shock, an estimated 1–5% of the external concentration of the peptide is present in the cytoplasm⁹. Cells were then plated on vitronectin-coated coverslip dishes, stimulated, fixed and ruptured as described in Fig. 1 legend before incubation with fluorescent phalloidin and antibodies.

FIG. 3 Confocal imaging of $\beta 3$ integrin in neutrophils plated on vitronectin. In confocal projections of permeabilized neutrophils, αv is seen at the leading edge of unbuffered cells and in small vesicles throughout the cell (a, c). Very few small vesicles are seen in $[Ca^{2+}]_i$ -buffered cells, and nearly all of the αv appears in clusters on the rear adherent surface of the cell (b, d). Control cells incubated with an irrelevant monoclonal antibody showed no staining with secondary antibody (not shown). Scale bar, 2 μm .

METHODS. Where indicated, cells were incubated in a solution of 50 μM $[Ca^{2+}]_i$ buffer QUIN-2/AM (ref. 2) for 40 min. Cells were incubated in the monoclonal antibody LM142 (5 μg ml^{-1}) (from D. Cheresh, Scripps Research Institute) and 10 nM fMLP for 1 h at room temperature, plated on vitronectin-coated coverslips and stimulated once again with 10 nM fMLP. Cells were fixed with 4% (v/v) paraformaldehyde for 4 min, and incubated with immunostaining buffer. The samples were incubated in a 1 μg ml^{-1} dilution of goat anti-mouse IgG (Pierce Scientific, Rockford, IL, USA) to block receptors on the upper surface of the cell, and then permeabilized with 250 μg ml^{-1} saponin in PBS. The cells were stained with a 1 μg ml^{-1} dilution of rhodamine goat anti-mouse IgG (Pierce Scientific) in blocking buffer, and rinsed thoroughly before viewing. Cells were visualized as previously described²⁸ on a Bio-Rad MRC600 laser scanning confocal microscope (Bio-Rad Microscience,



Cambridge, MA, USA) and 0.5- μm vertical sections were obtained. Three-dimensional images were then rendered with Microvoxel software (Indec Systems, Capitola, CA, USA), after passing through a $3 \times 3 \times 3$ gaussian convolution filter to reduce noise.

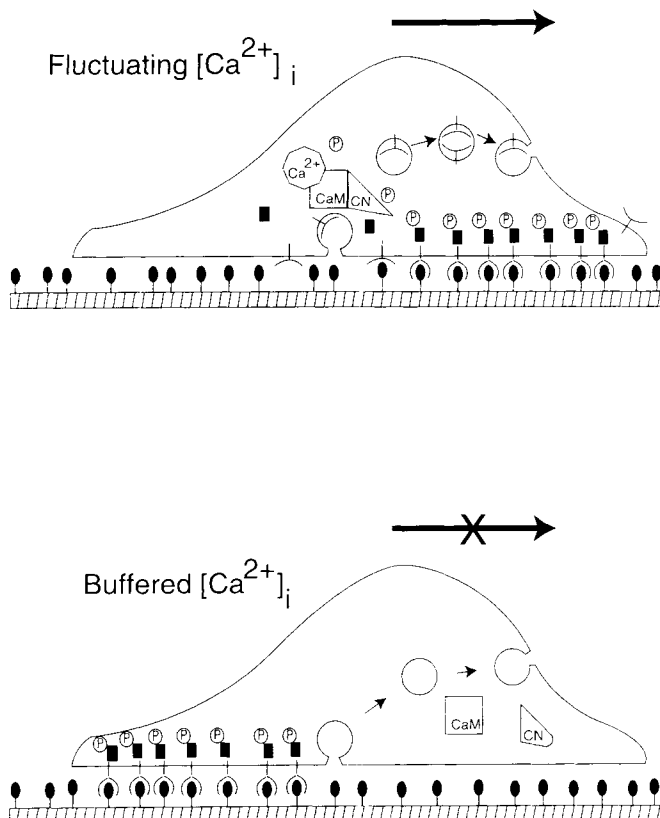


FIG. 4 Diagram of calcium regulation of neutrophil motility on vitronectin. $\alpha v \beta 3$ Integrins (cup shapes on stems) at the leading edge bind to vitronectin (filled ovals on stems), generating the traction needed for forward motion. The leading edge is also rich in actin and actin-binding proteins that are involved in linking the integrin with the cytoskeleton. During migration the body of the cell moves forward over the adhesion complex. Transient increases in $[Ca^{2+}]_i$ activate calcineurin (CN), which dephosphorylates an unidentified substrate (filled rectangles) in the adhesion complex allowing the integrin to detach from the vitronectin. The integrin is then internalized into endocytotic vesicles and recycled forward to the leading edge of the cell.

threonine phosphorylation can affect integrin adhesiveness¹⁷, the substrates involved have not been identified¹⁸.

A model has been proposed in which integrins are endocytosed at the rear of the cell and are recycled forward^{19,21}. Many integrins, including $\alpha\text{v}\beta 3$, have been shown to be endocytosed²², and it has been shown that recycling receptors are preferentially inserted at the leading edge of migrating cells²³. It has been difficult to test this model because inhibitors of constitutive endocytotic recycling are nonselective²⁴. Because $\alpha\text{v}\beta 3$ integrins on neutrophils require a specific signalling mechanism to be released from the substrate and endocytosed, we could selectively block this process.

To study endocytosis of $\alpha\text{v}\beta 3$ integrins, we used a monoclonal antibody that binds to αv without blocking binding to vitronectin²⁵. (Preincubation with this antibody does not affect the distribution of $\beta 3$ integrins in the lower adherent membrane of control or $[\text{Ca}^{2+}]_i$ -buffered cells.) Neutrophils were incubated in suspension with the antibody, plated on vitronectin and then stimulated to migrate. After 5 min the cells were fixed, permeabilized and labelled with a fluorescent secondary antibody. Figure 3a, c shows top and side views of a migrating neutrophil. The αv integrin is found in numerous vesicles throughout the cell. These vesicles may correspond to the specific granules that were shown previously to contain a vitronectin receptor²⁶. Because the primary antibody was initially bound to the surface of intact cells, these integrins must have been endocytosed into the cell.

When $[\text{Ca}^{2+}]_i$ was buffered, the integrins were found clustered toward the rear of the cell on the lower surface (Fig. 3b, d). The low level of intracellular staining in the $[\text{Ca}^{2+}]_i$ -buffered cells shows that essentially all of the recycling integrins had become trapped on the lower surface during the 5 min that the cells were attached to vitronectin. In separate experiments (not shown), $\beta 3$ integrins were localized in control or $[\text{Ca}^{2+}]_i$ -buffered cells by fixing the cells, permeabilizing them and then labelling with antibodies to $\beta 3$ cytoplasmic domains and fluorescent secondary antibodies. The distribution of the total $\beta 3$ pool was similar in both cases to the distribution of labelled αv shown in Fig. 3. This indicates that essentially all of the $\alpha\text{v}\beta 3$ integrins are cycling between the surface and endosomes and that they become trapped on the adherent membrane when $[\text{Ca}^{2+}]_i$ is buffered.

As shown schematically in Fig. 4, preferential insertion of $\alpha\text{v}\beta 3$ integrins at the leading edge along with $[\text{Ca}^{2+}]_i$ -regulated detachment and clearance from the adherent cell surface provides a mechanism for creating a gradient of adhesive strength from the front to the rear of a migrating cell. The inability of neutrophils to migrate on adhesive substrates when this process is blocked provides a clear demonstration that in these cells recycling of integrins to the front of the cell is required for continued migration on vitronectin. □

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ACKNOWLEDGEMENTS. We thank B. Hendey, J. Mandeville, R. Ghosh and E. Marcantonio for discussions and L. Maxfield for assistance with graphics; also C. Klee (NIH) for providing calcineurin inhibitory peptide and D. Cheresch (Scripps Research Institute) for antibodies. This work was supported by grants from the NIH.

Mortality before and after HIV infection in the complete UK population of haemophiliacs

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DURING 1977–91, 6,278 males diagnosed with haemophilia were living in the UK. During 1979–86, 1,227 were infected with the human immunodeficiency virus (HIV-1) as a result of transfusion therapy (median estimated seroconversion date, October 1982). Among 2,448 with severe haemophilia, the annual death rate was stable at 8 per 1,000 during 1977–84; during 1985–92 death rates remained at 8 per 1,000 among HIV-seronegative patients but rose steeply in seropositive patients, reaching 81 per 1,000 in 1991–92. Among 3,830 with mild or moderate haemophilia, the pattern was similar, with an initial death rate of 4 per 1,000 in 1977–84, rising to 85 per 1,000 in 1991–92 in seropositive patients. During 1985–92, there were 403 deaths in HIV seropositive patients, whereas 60 would have been predicted from rates in seronegatives, suggesting that 85% of the deaths in seropositive patients were due to HIV infection. Most of the excess deaths were certified as due to AIDS or to conditions recognized as being associated with AIDS.

Since 1976 the UK National Haemophilia Register¹ has included all UK residents diagnosed with haemophilia A (classical haemophilia, factor VIII deficiency) or haemophilia B (Christmas disease, factor IX deficiency). During 1977–91, 2,448 males with severe haemophilia, and 3,830 males with moderate or mild haemophilia were included in the Register and, on 1 January 1993, 82% were alive, 15% had died and 3% were lost to follow-up (Table 1).

During 1979–86, blood products used to treat haemophilia carried a risk of HIV-1 infection, and 4,043 patients (2,037 severe, 2,006 moderate or mild) are recorded as having received

‡ The investigators contributing to this study were M. Adelman, S. Al-Ismael, A. Aronstam, B. Atcock, T. Baglin, O. Baugh, J. Beard, J. Behrens, D. Bevan, P. Bevan, A. Black, A. Bloom, P. Bolton-Maggs, M. Boots, P. Caccia, C. Carter, J. Chandler, P. Chipping, M. Chisholm, H. Cohen, B. Colvin, J. Coppelstone, C. Costello, E. Craven, H. Daley, A. Dawson, G. Dolan, J. Dudley, S. Fairham, B. Gibson, D. Gillett, D. Goff, D. Gorst, P. Gover, P. Green, H. Hambley, P. Hamilton, I. Hann, P. Harper, C. Hay, J. Hayes, F. Hill, M. Howard, D. Howes, R. Ibbotson, R. Janmohamed, P. Jones, M. Kenny, P. Kernoff, D. King, H. Korn, J. Kramer, A. Kruger, M. Laffan, A. Laurie, M. Layton, C. Lee, R. Lee, J. Leslie, J. Lilleyman, G. Lowe, C. Ludlam, S. Machin, P. Mackie, J. Maitland, W. Mavor, E. Mayne, S. Mayne, M. McEvoy, P. McHugh, B. McVerry, E. Miller, M. Mills, D. Mitchell, V. Mitchell, E. Moffat, B. Murphy, N. Mir, D. Newsome, H. O'Brien, M. O'Shea, D. Osler, V. Oxley, L. Parapia, H. Parry, A. Patel, M. Phillips, C. Pollard, D. Prangnell, A. Prentice, E. Preston, C. Reid, C. Rist, J. Ross, G. Savidge, G. Scott, M. Semple, M. Shields, J. Shirley, C. Simpson, R. Stevens, R. Stockley, M. Streven, C. Taylor, T. Taylor, J. Thomas, D. Thompson, E. Thompson, R. Vaughan Jones, I. Walker, N. West, J. Wilde, M. Winter & A. Worsley.

Received 20 April; accepted 21 July 1995.

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TABLE 1 Males included in the UK National Haemophilia Register, 1977–91, by severity of haemophilia, HIV-test status and vital status

Vital status on 1 January 1993	Severe haemophiliacs*		Moderate or mild haemophiliacs†		All patients
	Tested seropositive for HIV		Tested seropositive for HIV		
	No	Yes	No	Yes	
Alive and living in the UK	1,195 (84%)	673 (66%)	3,132 (86%)	135 (65%)	5,135 (82%)
Dead	198 (14%)	341 (33%)	326 (9%)	72 (35%)	937 (15%)
Emigrated	2 (0.1%)	6 (0.6%)	16 (0.4%)	0 (—)	24 (0.4%)
Lost to follow-up	33 (2%)	0 (—)	149 (4%)	0 (—)	182 (3%)
Total	1,428 (100%)	1,020 (100%)	3,623 (100%)	207 (100%)	6,278 (100%)

Several smaller haemophilia cohorts from the UK reported previously are included in the present study^{16–19,23,24}. Vital status was ascertained from individual Haemophilia Centres and the National Health Service Central Registers. For each person a 'date last seen' was established. For those lost to follow-up this was the date of last contact with a Haemophilia Centre, whereas for other patients it was the earliest of: date of death, date of emigration, or 1 January 1993. HIV test results were collected in a series of annual surveys starting in 1985 (ref. 3). For 441 patients who were tested and found to be seropositive, the results of a previous seronegative test were available. Patients diagnosed or treated in the UK but living abroad are excluded, as are the few female patients. In addition, two severely affected patients (including one who had been tested seropositive for HIV) and two moderately affected patients, whose years of registration were after they had died, were excluded.

* Factor VIII or IX level of less than two international units per dl.

† Includes 104 patients with unknown severity, two of whom were tested seropositive for HIV.

potentially infected treatments. A reliable test for HIV antibodies² became available to Haemophilia Centres early in 1985. Among those who were alive on 1 January 1985, 78% of potentially infected severe patients and 52% of moderate/mild patients had been tested by December 1985, rising to 90 and 74% respectively by January 1993. One thousand and twenty severe patients and 207 moderate/mild patients were found to be infected with HIV (described as tested seropositive) (Table 1). For many patients, stored serum samples enabled the seroconversion date to be estimated reasonably precisely^{3,5}. The median estimated date of seroconversion was October 1982 for severe patients (range, June 1979–October 1986) and December 1982 for moderate/mild patients (range, October 1979–March 1986).

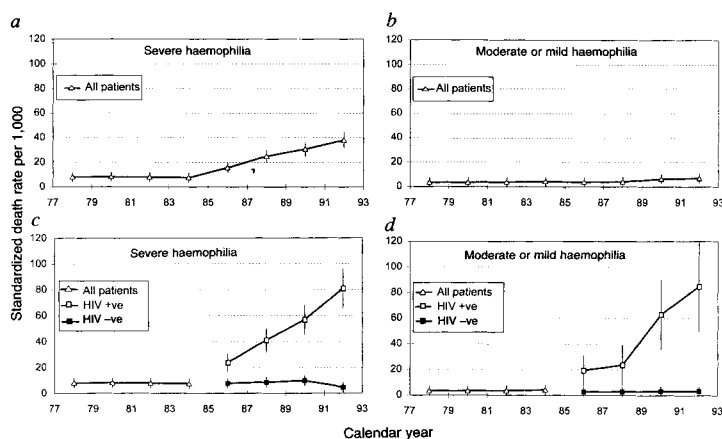
The annual death rate in patients with severe haemophilia remained steady at 8 per 1,000 during 1977–84, but then rose progressively to 38 in 1991–92 (Fig. 1a). This increase was confined to patients who tested seropositive for HIV and among whom the death rate increased steeply from 1985, reaching 81 in 1991–92; but in patients not tested as seropositive, the death rate during 1985–92 was 8 per 1,000, much as during 1977–84 (Fig. 1c). Among moderate/mild patients, the death rate during 1985–92 was 5 per 1,000, much as its value of 4 per 1,000 during 1977–84 (Fig. 1b). However, when HIV-seropositive patients were considered separately, the death rate again rose steeply during 1985–92 (Fig. 1d). Death rates during 1985–92 for patients tested for HIV and found not to be infected (tested seronegative) were close to rates for patients of unknown HIV

status within each severity group (Table 2). Thus little, if any, HIV-associated mortality has gone undetected.

The severely affected haemophiliacs had a higher initial mortality rate and also received much more transfusion therapy than patients with moderate/mild haemophilia, yet the excess death rate associated with HIV seropositivity was similar in patients with severe and with mild/moderate haemophilia (Table 2). In both groups excess mortality associated with HIV seropositivity increased progressively with time, the rates being 19, 34, 53 and 76 per 1,000 in the periods 1985–86, 1987–88, 1989–90 and 1991–92, respectively, for both groups combined (95% confidence intervals (CIs): 13–26, 26–42, 43–63, 63–89). Treatment, by prophylaxis against *Pneumocystis carinii* pneumonia⁶ or with zidovudine^{7,22}, has been widespread for HIV-infected haemophiliacs since about 1989. However, the steady increase in the excess death rate from 1985 to 1992 suggests that in this population the increasing impact of HIV-associated mortality has not been halted by these treatments. This study includes deaths only to 1992, and so does not permit examination of data following widespread use in the UK of high purity factor concentrate⁸.

Use of the certified cause of death allows comparison of mortality rates from specific causes with those for the nation as a whole. Among patients with severe haemophilia who were not tested seropositive for HIV, there were significant increases in mortality during 1985–92 from coagulation defects, intracranial haemorrhage, injury, poisoning and suicide, and from hepatitis, liver disease and primary liver cancer, which are associated with chronic hepatic infections (Table 3). For all these causes com-

FIG. 1. Annual death rates per 1,000, directly standardized for age, and 95% confidence intervals, by calendar year and severity of haemophilia. Panels a and b give values for all patients in each severity group. Panels c and d give separate values for HIV seropositive patients and patients not known to be HIV seropositive from 1985. Rates were obtained by calculating death rates (ratio of observed deaths to person-years at risk) in age-groups <15, 15–24, 25–34, 35–44, 45–54, 55–64, 65–84 by calendar period (1977–78, 1979–80, ..., 1991–92). Person-years at risk were calculated by considering the length of time from registration to the date last seen (see Table 1) for each patient. Observed deaths and person-years over age 84 were excluded. After early 1985, patients becoming ill are likely to have been tested. Therefore, here and in Tables 2 and 3, patients tested seropositive for HIV with estimated seroconversion dates before 1 January 1985 are counted as seropositive from 1 January 1985, while the 93 patients with estimated seroconversion dates on or after 1 January 1985 contribute to the group of those not tested seropositive until their date of seroconversion, estimated as in ref. 4. For age-standardization, a weighted average of the age-specific death rates was calculated, with weights proportional to the total



number of person-years at risk in the HIV seropositive patients in the whole period 1985–92 for both severity groups combined. Confidence intervals were calculated using the normal approximation.

TABLE 2 Observed deaths, annual death rates per 1,000, and annual excess death rates per 1,000 in HIV-seropositive haemophiliacs, by severity of haemophilia and calendar period

Patients with severe haemophilia									
All severely affected patients			HIV status						Excess death rate in seropositive patients*
			Unknown		Seronegative		Seropositive		
	O§	Death rate	O	Death rate	O	Death rate	O	Death rate	
1977–78	25	7.9	25	—	0	—	0	—	—
1979–80	30	8.1	30	—	0	—	0	—	—
1981–82	31	7.9	31	—	0	—	0	—	—
1983–84	30	7.5	23	—	0	—	7†	—	—
1977–84	116	7.9 (6.4–9.4)‡	—	—	—	—	—	—	—
1985–86	66	15.6	15	6.2	8	15.0	43	23.9	16.2
1987–88	98	25.0	11	8.2	13	9.3	74	41.3	33.6
1989–90	118	30.7	9	10.0	13	9.9	96	56.8	49.2
1991–92	136	37.9	8	6.1	7	3.6	121	80.8	73.2
1985–92	418	27.1 (24.4–29.8)	43	7.3 (4.7–9.8)	41	8.1 (5.4–10.9)	334	49.1 (43.7–54.4)	41.4 (35.8–47.0)

Patients with moderate or mild haemophilia									
All moderately or mildly affected patients			HIV status						Excess death rate in seropositive patients*
			Unknown		Seronegative		Seropositive		
	O	Death rate	O	Death rate	O	Death rate	O	Death rate	
1977–78	21	3.4	21	—	0	—	0	—	—
1979–80	28	3.5	28	—	0	—	0	—	—
1981–82	33	3.6	33	—	0	—	0	—	—
1983–84	50	4.2	46	—	1†	—	3†	—	—
1977–84	132	3.7 (3.0–4.5)	—	—	—	—	—	—	—
1985–86	49	3.8	31	2.8	5	2.4	13	19.4	16.3
1987–88	46	4.1	22	3.4	14	2.0	10	23.8	20.6
1989–90	69	6.5	28	2.4	19	4.6	22	63.0	59.9
1991–92	78	6.9	28	2.8	26	4.1	24	84.7	81.6
1985–92	242	5.4 (4.5–6.3)	109	2.9 (2.2–3.6)	64	3.5 (2.3–4.6)	69	45.2 (33.7–56.7)	42.1 (30.6–53.6)

Death rates calculated and age-standardized and confidence intervals calculated as for Fig. 1. Observed deaths and person-years over age 84 were excluded. Separate death rates for: (1) patients tested seronegative for HIV, and (2) patients of unknown HIV status, calculated by subdividing observed deaths and person-years during 1985–92 among those not tested seropositive for HIV into those who tested seronegative, with no potentially infected treatments recorded during the same or a subsequent calendar year, and others.

* Excess death rates obtained by subtracting from each calendar period-, age- and severity-specific death rate for HIV-seropositive patients, the corresponding age- and severity-specific rate for seronegative patients and patients of unknown serostatus combined, 1985–92. Age standardization and confidence intervals for excess death rates calculated as in Fig. 1.

† HIV testing was not generally available before 1985. Although some patients dying before this were tested, testing was not carried out retrospectively for the majority of patients who died. Therefore death rates by HIV status cannot be calculated before 1985. The certified causes of death of the 10 known seropositive patients dying in 1983–84 were: haemophilia (2), suicide (2), cerebrovascular accident, cirrhosis, coronary thrombosis, diabetes mellitus, myocardial infarction, renal failure. None suggests immunodeficiency. Before 1985 only one death, in a patient not reported as tested for HIV by any Haemophilia Centre, was certified as due to AIDS.

‡ 95% confidence intervals in parentheses.

§ O, observed deaths.

bined (category B) the ratio of observed to national expected deaths (O/E) was 13.3 (95% CI 10.0–17.2). Most of these associations have been reported elsewhere for haemophiliacs^{9–15}. Ischaemic heart disease mortality was lower than expected, as in other haemophilia populations¹¹. For other causes, mortality was similar to that in the general population ($O/E=1.1$, 95% CI 0.7–1.7, category D). Patterns of cause-specific mortality for all patients with severe haemophilia during 1977–84 were similar (data not shown).

During 1985–92, 403 deaths occurred in seropositive patients and for 235 of these the certified cause was AIDS (ICD-9 code 279.1; Table 3). For the remaining 168 deaths in HIV-seropositives, there were significant excesses for many causes indicative of AIDS, including infections, non-Hodgkin's lymphoma and pneumonia, and also significant excesses for causes associated with haemophilia. Information received from the

Haemophilia Centres indicates that many of these patients had in fact developed AIDS, indicating that in AIDS patients there is a tendency to attribute cause of death to diseases associated with haemophilia or AIDS rather than to AIDS itself. However, not all the excess mortality in patients tested seropositive for HIV appears to be due to recognized AIDS indicator diseases, and some may be due to other conditions such as liver disease.

The UK National Haemophilia Register data provide a unique opportunity to examine the impact of HIV-1 infection in a complete population where almost all potentially infected individuals have been tested. These are the first data to document that, in a large and complete population, mortality among those who by chance were infected with HIV increased more than tenfold while remaining unchanged over time in those who escaped infection (Fig. 1c, d and Table 2). Assuming that the

TABLE 3 Cause-specific mortality during 1985–92 by HIV status compared with national mortality

Certified cause of death (ICD-9 codes)	Tested seropositive for HIV					
	O [†]	No* E§	O/E	O	Yes [†] E	O/E
(A) AIDS, HIV, etc. (279.1)	0	0.10	0.0	235	0.12	1,958.3***
(B) Causes significantly increased in severe haemophilia without HIV						
Hepatitis and liver disease (070, 570–573)	6	0.37	16.2***	11	0.30	37.0***
Liver cancer (155.0–155.1)	2	0.11	18.7*	1	0.07	15.1
Coagulation defects, etc. (280–289)	33	0.11	307.2***	72	0.06	1,155.7***
Intracranial haemorrhage (ICH, 430–432)	5	0.49	10.2***	1	0.37	2.7
Injury, poisoning and suicide (E800–999)	10	3.14	3.2**	8	3.68	2.2
All causes in category (B)	56	4.21	13.3***	93	4.47	20.8***
(C) Ischaemic heart disease (IHD, 410–414)	5	10.37	0.5	5	5.74	0.9
(D) Other causes						
Infections excl. hepatitis (001–139, excl. 070)	0	0.23	0.0	11	0.14	75.6***
Hodgkin's disease (201)	0	0.06	0.0	2¶	0.07	29.1**
Non-Hodgkin's lymphoma (200, 202)	0	0.29	0.0	12	0.21	57.1***
Other neoplasms excl. liver (140–239 excl. 155.0–1 and 200–2)	9	9.38	1.0	7#	5.28	1.3
Endocrine disorders excl. AIDS, HIV, etc. (240–279 excl. 279.1)	1	0.50	2.0	1	0.30	3.3
Mental disorders (290–319)	0	0.35	0.0	2	0.25	8.1
Nervous system incl. dementia (320–389)	1	0.78	1.3	6	0.54	11.2***
Circulatory excl. IHD and ICH (390–459 excl. 410–4 and 430–2)	7	3.86	1.8	6	1.85	3.2*
Pneumonia (480–486)	0	0.63	0.0	12	0.31	38.6***
Other respiratory (rest of 460–519)	2	2.14	0.9	2	0.99	2.0
Digestive system excl. liver (520–579 excl. 570–573)	0	0.63	0.0	3	0.34	8.7*
Musculoskeletal and connective tissue (710–739)	1	0.11	9.0	2	0.06	33.8*
Other diseases (580–709, 740–799)	1	1.03	1.0	2**	0.36	5.5
All causes in category (D)	22	20.00	1.1	68	10.70	6.4***
(E) Death certificate not located	1	—	—	2	—	—
All causes	84	34.68	2.4***	403	21.03	19.2***

Death details obtained from the Office of Population Censuses and Surveys (OPCS) or the General Register Offices (GRO) in Edinburgh or Belfast. Underlying cause coded to the ninth revision of the International Classification of Diseases (ICD-9)²⁰ by OPCS. The final certified cause may differ from that available to the public if the certifier indicates that further information may become available, and later supplies this confidentially. This system, little used in the 1970s, is commonly used for HIV-related disease²¹. The numbers of deaths with final certified cause in each category were obtained from OPCS and the GROs and the number of deaths certified to code 279.1 increased by 47, from 188 to 235 for patients tested seropositive for HIV and remained at zero for patients not tested seropositive. Observed and expected deaths over age 84 are excluded.

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (two-sided Poisson test).

* Patients with severe haemophilia not tested seropositive for HIV.

† Patients with severe or moderate/mild haemophilia tested seropositive for HIV. 83% of person-years are for patients with severe haemophilia.

‡ O, observed deaths.

§ E, expected deaths from national rates, calculated by multiplying the number of person-years in each calendar year and 5-year age group by the corresponding death rate for males in England and Wales.

|| Septicaemia, viral encephalitis, herpes zoster, retrovirus infection (2), toxoplasmosis (4), *Pneumocystis* (2). All suggest immunodeficiency, except possibly septicaemia.

¶ Review of the clinical notes for one of these patients showed that he had a cerebral non-Hodgkin's lymphoma as well as Hodgkin's disease.

One case each of carcinoma of bronchus, duodenum, colon, rectum, pancreas, osteosarcoma and neurofibromatosis.

** For both deaths the certificate indicated that the cause was unknown.

death rate during 1985–92 among infected patients would, in the absence of HIV, have been close to that for uninfected patients, 60 deaths would have been predicted, whereas 403 deaths in fact occurred, an excess of 343. Thus 85% of the deaths in HIV seropositive patients are likely to have been caused by HIV. This large excess, together with the temporal pattern of the increase

in those who became infected, the similarity of the excess death rate associated with HIV infection regardless of the severity of haemophilia, and the large increase in mortality from conditions not usually associated with haemophilia, demonstrate particularly clearly the enormity and the specificity of the effect of HIV-1 infection on mortality in this population. □

Received 21 April; accepted 17 August 1995.

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ACKNOWLEDGEMENTS. We thank the Office of Population Censuses and Surveys and the General Register Offices in Edinburgh and Belfast for their help in tracing the population and providing death details; A. McCormick, J. Arrundale and L. McKeag for assistance in obtaining the final certified cause of death; P. Wallace and L. Cutler for clerical and secretarial work; and many friends and colleagues for advice. This project is supported by the MRC, the Imperial Cancer Research Fund, and the Oxford Haemophilia Centre.

Requirement of pointed-end capping by tropomodulin to maintain actin filament length in embryonic chick cardiac myocytes

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CONTROL of actin filament length and dynamics is important for cell motility and architecture and is regulated in part by capping proteins that block elongation and depolymerization at both the fast-growing (barbed) and slow-growing (pointed) ends¹⁻⁴. Tropomodulin is a capping protein for the pointed end of the actin filament^{5,6}; it is associated with the free, pointed ends of the thin filaments in striated muscle, where it is thought to bind to both tropomyosin and actin^{7,8}. In embryonic chick cardiac myocytes, tropomodulin assembles after the thin, as well as the thick, filaments have become organized into periodic I and A bands⁸, suggesting that tropomodulin might be involved in maintaining actin filament length. Here we show that microinjection of an antibody that inhibits tropomodulin's pointed-end-capping activity *in vitro* results in a marked elongation of actin filaments from their pointed ends and a >80% reduction in the percentage of beating cells. This demonstrates that pointed-end capping by tropomodulin is required to maintain actin filament length *in vivo* and that this is essential for contractile function in embryonic chick cardiac myocytes.

We generated a monoclonal antibody (mAb9) to recombinant chick skeletal muscle tropomodulin that specifically recognizes tropomodulin in extracts of embryonic chick cardiac myocytes (Fig. 1a). This antibody binds to an epitope in the carboxy-terminal half of tropomodulin (residues 190-359), as demonstrated by western blotting with various recombinant tropomodulin fragments (Fig. 1b, c). MAb9 has no effect on the binding of tropomodulin to tropomyosin (results not shown), consistent with the amino-terminal half of tropomodulin (residues 6-184) containing the binding sites for tropomyosins⁹.

We found that mAb9 inhibits the ability of tropomodulin to cap the pointed ends of tropomyosin-actin filaments *in vitro*. Pointed-end capping was evaluated from the inhibition of elongation and depolymerization of tropomyosin-actin filaments that were copolymerized in the presence of gelsolin; polymerization was measured by fluorescence changes of pyrenyl actin. Gelsolin was used to cap the barbed filament end because we were interested in events occurring exclusively at the pointed end. Figure 2a, b demonstrates that preincubation of tropomodulin with an excess of mAb9 completely reverses the ability of tropomodulin to inhibit elongation as well as depolymerization from the pointed ends of tropomyosin-actin filaments. This is due to the effect of mAb9 on the specific interaction of tropomodulin with actin, because mAb9 also abolishes the ability of tropomodulin to inhibit elongation from the pointed ends of pure actin filaments in the absence of tropomyosin (Fig. 2c). These results suggest that the C-terminal domain of tropomodulin is essential for its capping activity, presumably because it contains the actin-binding site. On the other hand, binding of tropomodulin to tropomyosin alone, which is not affected by mAb9 (see above), is not sufficient for pointed-end capping.

We microinjected mAb9 into embryonic chick cardiac myocytes to determine whether inhibition of tropomodulin's actin filament pointed-end capping activity would lead to alterations in actin filament length and cell function *in vivo*. Fluorescence staining for actin filaments 1 hour after microinjection with mAb9 revealed a dramatic loss of actin striations in many myofibrils (Fig. 3b, long arrows; note gaps in staining in some non-striated myofibrils (small arrow)). But the normal striated actin-staining pattern remained unchanged in cells injected with control mouse IgG (Fig. 3c, d). The loss of actin striations in mAb9-microinjected cells is not due to contraction of myofibrils and juxtaposition (or overlapping) of adjacent sets of actin filaments in each sarcomere, because the normal periodicity of tropomyosin striations was not altered in these myofibrils (Fig. 3i-l, arrows). The appearance of α -actinin, myosin and titin striations also remained unaltered when examined by immunofluorescence staining (results not shown). Therefore we conclude that the continuous, non-striated regions of actin-filament staining along myofibrils in mAb9-injected cells are a consequence of specific *de novo* elongation of actin filaments from their pointed ends across the gap in the middle of the sarcomere (H zone).

Despite the elongation of actin filaments from their pointed ends in the mAb9-microinjected cells, tropomodulin retained its original position in the myofibrils as indicated by the striated staining pattern for the microinjected mAb9 (Fig. 3a; see ref. 8). Presumably, tropomodulin remained bound to tropomyosin, consistent with the *in vitro* data that mAb9 blocks tropomodulin

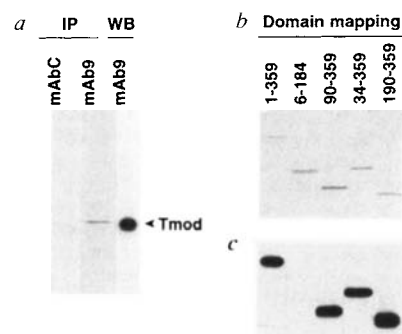


FIG. 1 mAb9 recognizes an epitope within the C-terminal half of tropomodulin. a, mAb9, but not control mouse IgG, specifically immunoprecipitates tropomodulin (lanes 1 and 2) from [³⁵S]methionine-labelled extracts and also recognizes tropomodulin on western blots (lane 3) of embryonic chick cardiomyocyte cultures. b, Coomassie-blue-stained gel. c, Western blot of recombinant skeletal muscle tropomodulin and tropomodulin fragments probed with mAb9, followed by rabbit anti-mouse IgG and [¹²⁵I]-labelled protein A. mAb9 recognizes the tropomodulin fragments containing residues 34-359, 90-359 and 190-359 encompassing the C-terminal domain, but not the N-terminal domain containing residues 6-184. Abbreviation: Tmod, tropomodulin.

METHODS. Full-length (1-359) recombinant tropomodulin and fragments were expressed and purified as described previously⁹. 0.61 pmol of purified protein was electrophoresed on a 12% SDS-polyacrylamide mini-gel, transferred to nitrocellulose, blocked in 4% BSA in PBS, and sequentially probed with 10 μ g ml⁻¹ mAb9, rabbit anti-mouse IgG (Pierce, Rockford, IL) (diluted 1:500) and [¹²⁵I]-labelled protein A as described¹⁰. The amino acids included in each fragment are indicated. Each fragment has an additional 15 amino acids at the N terminus from the glutathione S-transferase linker region⁹. MAb9 was generated by immunization and screening with recombinant chicken skeletal muscle tropomodulin (V.M.F. and C. K. Grant, manuscript in preparation) (Custom Monoclonals, W. Sacramento, CA). Antibodies were purified over a protein-G-Sepharose column. Metabolic labelling of cardiac myocytes with Tran[³⁵S]-label (ICN Pharmaceuticals, Irvine, CA), preparation of extracts and immunoprecipitation were performed as described previously⁸.

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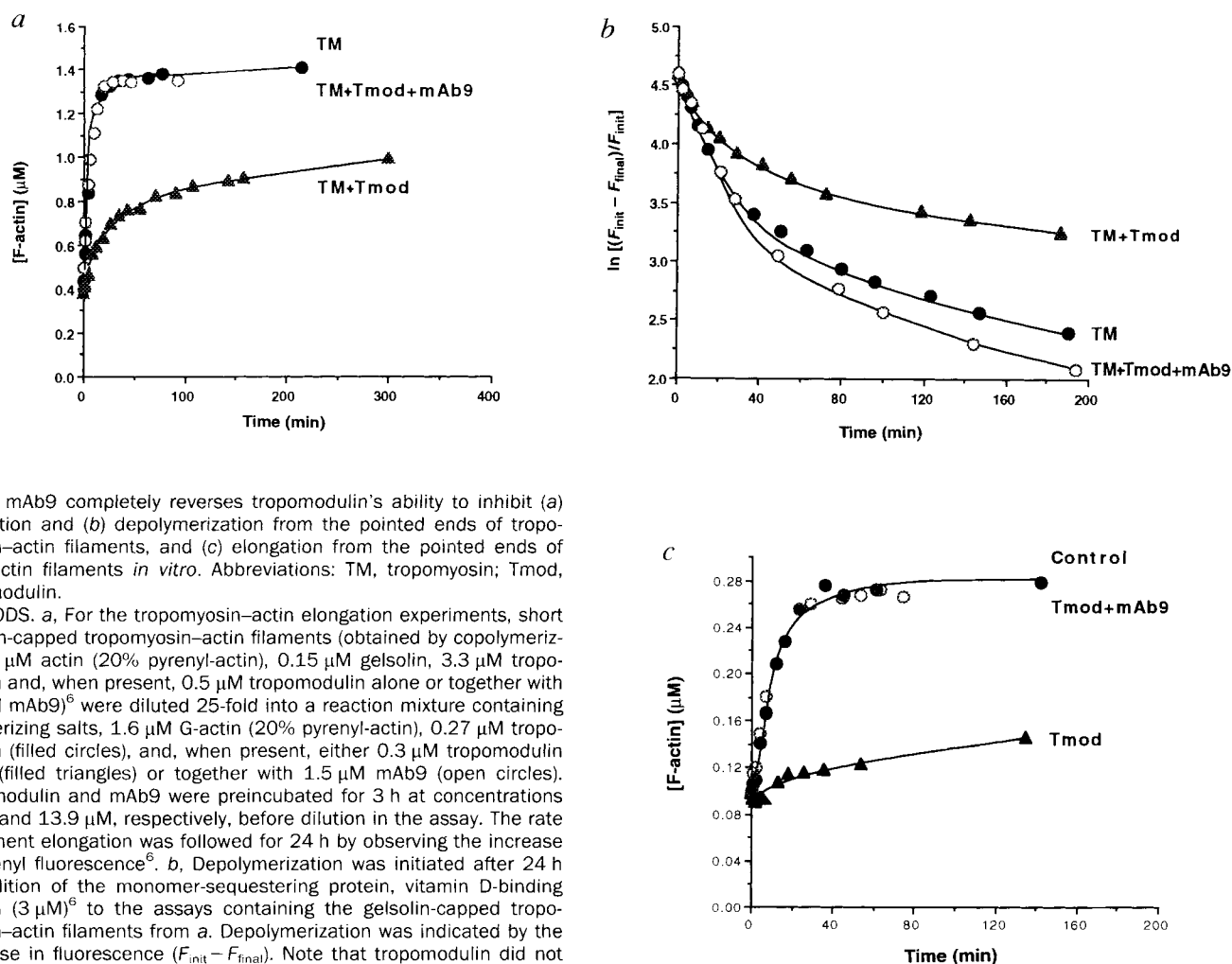


FIG. 2 mAb9 completely reverses tropomodulin's ability to inhibit (a) elongation and (b) depolymerization from the pointed ends of tropomyosin-actin filaments, and (c) elongation from the pointed ends of pure actin filaments *in vitro*. Abbreviations: TM, tropomyosin; Tmod, tropomodulin.

METHODS. a, For the tropomyosin-actin elongation experiments, short gelsolin-capped tropomyosin-actin filaments (obtained by copolymerizing 10 μM actin (20% pyrenyl-actin), 0.15 μM gelsolin, 3.3 μM tropomyosin and, when present, 0.5 μM tropomodulin alone or together with 2.0 μM mAb9)⁶ were diluted 25-fold into a reaction mixture containing polymerizing salts, 1.6 μM G-actin (20% pyrenyl-actin), 0.27 μM tropomyosin (filled circles), and, when present, either 0.3 μM tropomodulin alone (filled triangles) or together with 1.5 μM mAb9 (open circles). Tropomodulin and mAb9 were preincubated for 3 h at concentrations of 2.8 and 13.9 μM, respectively, before dilution in the assay. The rate of filament elongation was followed for 24 h by observing the increase in pyrenyl fluorescence⁶. b, Depolymerization was initiated after 24 h by addition of the monomer-sequestering protein, vitamin D-binding protein (3 μM)⁶ to the assays containing the gelsolin-capped tropomyosin-actin filaments from a. Depolymerization was indicated by the decrease in fluorescence ($F_{init} - F_{final}$). Note that tropomodulin did not completely inhibit elongation and depolymerization of tropomyosin-actin filaments as shown previously⁶. This may be due to a difference between the chicken breast muscle tropomyosin used here, which consisted primarily of α,α homodimers, and the rabbit skeletal tropomyosin used previously⁶, which contained α and β homo- and heterodimers (V.M.F., unpublished observations). c, The elongation measurements

using pure actin were made as above except with lower G-actin concentrations (0.8 μM), more tropomodulin (0.5 μM) and less mAb9 (1.0 μM)⁶. Filled circles; actin alone (control); filled triangles, in the presence of tropomodulin; open circles, in the presence of Tmod and mAb9.

binding to actin without affecting tropomodulin binding to tropomyosin (see above). In addition, the normal striated appearance of tropomyosin staining in cells microinjected with mAb9 indicated that no tropomyosin was associated with the newly elongated actin filaments (Fig. 3i-l). It is possible that binding of tropomodulin to the terminal tropomyosin molecules on the thin filaments may prevent further elongation of the tropomyosin polymer^{7,10}. Self-nucleation of new tropomyosin polymers on the actin filament extensions is unlikely to take place, owing to the very low concentrations of free tropomyosin in cardiac myocytes^{8,11}.

The effect of mAb9 was reversible after its removal or degradation by the cell. After 24 hours, in those cells where staining of the microinjected antibody had become barely detectable, the actin striations again closely resembled those found in uninjected cells and in cells injected with a control IgG (Fig. 3e, f, arrows). In contrast, in other cells in which the striated immunofluorescent staining pattern of the microinjected mAb9 still persisted, the actin staining had become completely non-striated and no gaps along any myofibrils could be discerned (Fig. 3g, h, arrows). The basis for the delayed inhibitory effect of mAb9 on

tropomodulin function at the pointed filament ends in some sarcomeres is not understood (for example, see Fig. 3b).

The actin filament extensions in mAb9-microinjected cells would be expected to be unregulated because the interaction of the troponin complex with tropomyosin on the thin filament is required for calcium regulation of contraction in striated muscle^{12,13}. Unregulated stretches of actin filaments are permanently 'on' with respect to interaction with myosin heads and cannot relax¹³. Therefore we investigated whether microinjection of mAb9 into cardiac myocytes might interfere with cell beating, which consists of cycles of contraction followed by relaxation in rapid succession. Using a video microscopy system we found that microinjection of mAb9 or Fab fragments of mAb9 led to a >80% reduction in the proportion of beating cells when compared with cells injected with buffer, control IgG or control IgG Fabs (Fig. 4). We conclude that when tropomodulin is inactivated, leading to the formation of unregulated actin filament extensions at the free (pointed) ends of the thin filaments, beating of cardiac myocytes is suppressed.

In conclusion, pointed-end capping of actin filaments by tropomodulin is essential to maintain the final lengths of thin fila-

FIG. 3 Microinjection of mAb9 results in a marked loss of actin striations, owing to elongation of actin filaments from their free (pointed) ends. Cells in day 5 or 6 chick cardiac myocyte cultures were microinjected with mAb9 (a, b, e–l) or a control antibody of the same isotype (IgG₁: mAbC) (c, d), and then incubated for 1 (a–d, i–l) or 24 (e–h) hours. The injected cells were stained with rhodamine-conjugated donkey anti-mouse IgG (Accurate Chemical and Scientific, Westbury, NY) to identify anti-tropomodulin IgG (a, e, g) or the control IgG (c) and bodipy-conjugated (where bodipy is 4,4-difluoro-4-bora-3a,4a-diaza-5-indacene) phalloidin (b, d, f, h) to stain actin filaments. To localize tropomyosin together with actin, cells were triple-labelled with rabbit anti-human erythrocyte tropomyosin antibody²⁰, followed by donkey anti-rabbit IgG (i, k), bodipy-phalloidin (j, l) and Cascade blue-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) to identify injected cells (not shown). For each experimental group >500 injected cells were observed. Microinjection of Fab fragments of mAb9 gave the same results as injection of the intact molecule (results not shown). No alterations in the distribution of actin filaments were detected in chicken embryonic fibroblasts microinjected with mAb9 (results not shown): this cell type does not contain any detectable tropomodulin⁸. Scale bar, 10 μ m.

METHODS. Cardiac myocytes were prepared and cultured as described⁹, except that the cells were grown on collagen-coated gridded coverslips (Bellco Glass, Vineland, NJ) (0.01% collagen type I (Sigma, St Louis, MO)). Cells were injected with a 10 mg ml⁻¹ solution of antibody in 10 mM Tris, pH 7.4, 25 mM KCl, with an Eppendorf injector system attached to a Zeiss axiovert microscope and processed for immunofluorescence microscopy as described⁸.

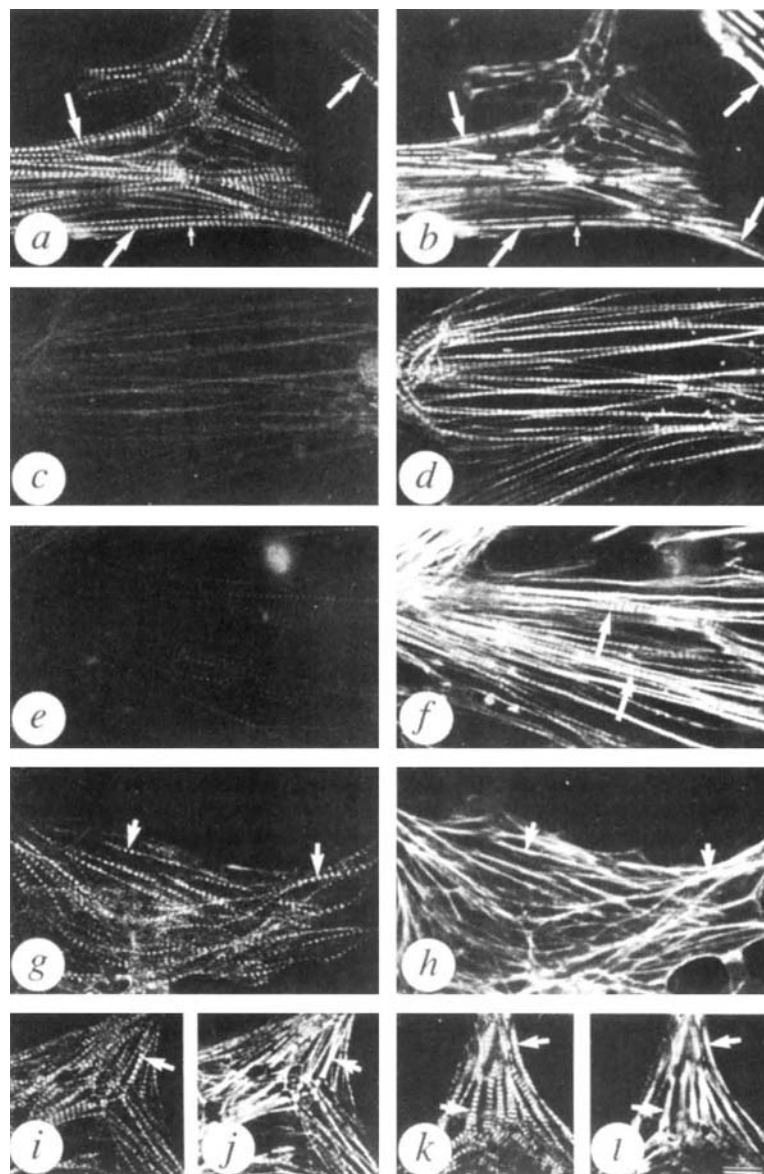
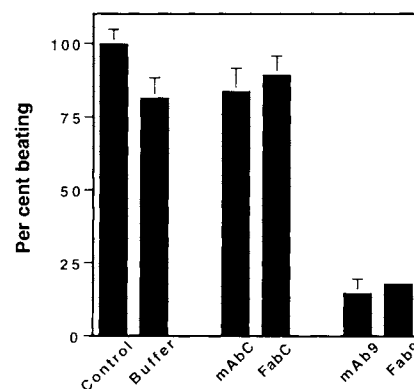


FIG. 4 Microinjection of mAb9 inhibits the contractile activity of cardiac myocytes. Control, no injection; Buffer, injection with buffer; mAbC, injection with a 1 mg ml⁻¹ control IgG; FabC, injection with 3.5 mg ml⁻¹ Fabs of the control antibody; mAb9, injection with 1 mg ml⁻¹ of anti-tropomodulin monoclonal antibody 9; Fab9, injection with 3.5 mg ml⁻¹ Fabs of mAb9. Values are the means of 2 (buffer, FabC, Fab9) or 3 (control, mAbC, mAb9) experiments $\pm$ s.d.

METHODS. Five- or six-day cultures were analysed for beating by identifying groups of ~400 cells at similar densities on gridded coverslips, projecting cells on a video screen (Zeiss camera model ZVS-47E, Sony Trinitron superfine-pitch monitor), recording the beating activity on a videotape recorder and counting the number of cells that were beating or not beating. After the initial recording, the cells were incubated for 1 h in medium containing 15 mM HEPES and 20 mM 2,3-butanedione monoxime (BDM; Sigma); this drug is a rapid, reversible inhibitor of cardiac contractility that is believed to minimize injury to cells during microinjection²¹. Each group of 400 cells was then injected, incubated for an additional hour in medium with BDM and HEPES, and then for a further 3 h in standard medium. The beating and non-beating cells were again recorded by video microscopy and counted. Because not all cells on a coverslip were beating, the number of cells beating after injection is given as a percentage of the cells beating before injection. In general, between 60 and 75% of the cells on a coverslip were beating



before injection. In some experiments, after recording the beating activity of the microinjected cells on videotape, the cells were fixed and stained for actin and mAb9 as described (see Fig. 3 legend). Direct comparison of identical cells showed that all mAb9-microinjected cells that had lost their striations were not beating.

ments *in vivo* as well as for contractile activity in embryonic chick cardiac myocytes. We speculate that pointed-end regulation by tropomodulin may play a role in controlling actin filament length and function in all cell types where it is expressed^{7,10,14,19}. □

Received 7 June; accepted 1 August 1995.

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ACKNOWLEDGEMENTS. We thank L. Gerace, S. Schmid and the members of the Fowler lab for suggestions and comments; G. Babcock for producing the tropomodulin fragments; P. Vogt for allowing us to use his microinjection/video microscopy system; C. Conely and M. Woo for help with the cover micrograph; and J. Sanger and the teaching staff of the Woods Hole short course in microinjection for advice on microinjection techniques. This work was supported by grants from the American Heart Association and the NIH to V.M.F. and by a grant from the NIH to A.W. C.C.G. is a recipient of a postdoctoral fellowship award from the California Affiliate of the American Heart Association.

CORRECTION

Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow

Ronen Alon, Daniel A. Hammer
& Timothy A. Springer

Nature **374**, 539–542 (1995)

WE wish to make a correction to this Letter. Reference 26 to work on the CD2-LFA-3 interaction should be replaced with van der Merwe, P. A. *et al. Biochemistry* **33**, 10149 (1994). A statement was omitted from the text referring to the reference cited as ref. 26 by Kaplanski *et al.* This should read 'Kaplanski *et al.* provided estimates of rapid on and off rates for E-selectin from analysis of pauses in neutrophil rolling on endothelial monolayers'. Although obtained in a less direct manner from a system complicated by existence of multiple E-selectin-ligand bonds at any one time, and non-E-selectin-dependent bonds, these were comparable to the kinetic constants we measured for P-selectin. □

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Fisons Instruments **isotopic analysis** (FIA) has been refined with VG Optima's software capability, expanding the sample preparation range and adding a remote diagnostic facility (Isolink) (*Reader Service No. 100*). This instrument offers sensitivity in a small-radius IRMS, with typical detection values as low as 1,000 molecules CO₂ per mass 44 ion. Other enhancements include an improved vacuum pumping system with ceramic-bearing, turbo-molecular pumps for increased reliability and extended performance. The user-programmable, OS/2 multi-tasking software can be customized to suit individual requirements and its compatibility with MS-DOS and Windows enables a variety of applications to be run simultaneously for increased flexibility.

EG&G offers **single-photon counting**, a technique used for quantitative measurements in chemistry, biochemistry, physics and the atmospheric sciences (*Reader Service No. 101*). The new eight-page application note, AN51 Pulse-Processing Electronics for Single-Photon Counting, with counting rates from 10¹ to 10⁷ counts per second, offers guidance to assembling the appropriate photon counting system for specific applications. The note describes methods and instrumentation suitable for single-photon counting with steady-state or time-variant light sources. Simple explanations of how the systems work are provided along with a summary of the precision available under extreme operating conditions. Examples with block diagrams are provided for DIAL, LIDAR, Raman spectroscopy and phosphorescence-decay applications.

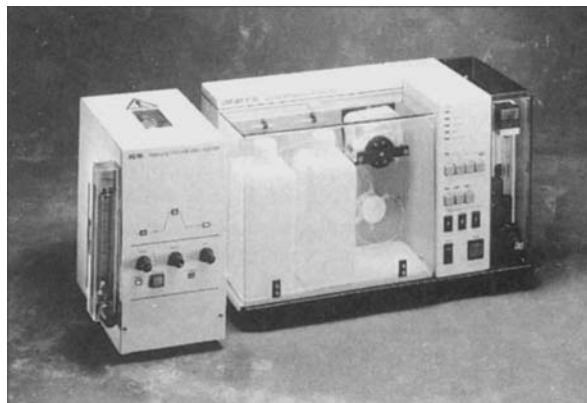
Finnigan brings its new LCQ LC/MS/MS to laboratories to perform ultra-trace analysis by **liquid chromatography/mass spectroscopy** (*Reader Service No. 102*). The new LCQ is a compact, benchtop system that offers high sensitivity, full-scan spectra and the selectivity of MS/MS. The LCQ combines the features of high-performance quadrupole

systems with the sensitivity of the ion trap. Two inlets are available, electrospray ionization and atmospheric pressure chemical ionization — both are atmospheric-pressure, ionization techniques. Positive and negative ion analysis are standard features, as is MSⁿ, a feature that should prove useful in pharmaceutical analysis and protein chemistry.

The new Shimadzu AA-6701 **atomic absorption spectrophotometer** with PC control in a Windows environment allows easy switching between flame and furnace analyses (*Reader Service No. 103*). This unit, with no manual hardware exchanges, provides easy switching between furnace and flame analysis via the PC keyboard. EPA-approved, self-reversal and deuterium-background correction are standard features. Standard and sample dilution, dilution and reanalysis of over-range samples, matrix modifications, calibration, switching from flame to furnace, optimization of gas flow and atomizer alignment, gas leakage detection, flame monitoring and flashback prevention are all automatic. Options available include auto-sampler for flame-continuous sampling and flame-furnace microsampling, hydride vapour generation and cold-mercury vapour generation.

Thermo Electron has launched a new generation of **high-performance bench-top EDXRF systems** (*Reader Service No. 104*). QuanX is designed to provide performance for the rapid and non-destructive EDXRF analysis of inorganic elements in soil, sludges, oils, dust, polymers and more. The small footprint and convenience of Peltier cooling added to the unique Si(Li) detector allow transportability in a mobile laboratory environment if required. A Windows operating interface and single-standard, fundamental parameters software simplifies operation and set up. On-line service is achieved through internal modem remote diagnostics.

To meet the demanding detection requirements in the field of **mercury determination**, ATI Unicam provides the HG90 mercury concentration system (*Reader Service No. 105*). The system is an accessory that allows the preconcentration of mercury utilizing the process of amalgam formation. Mercury vapour is passed for an accurately



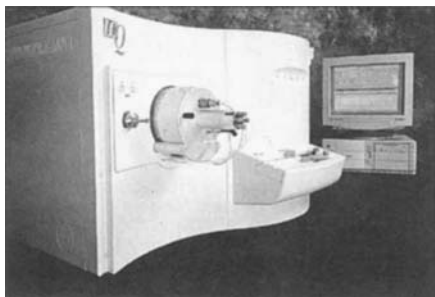
ATI Unicam HG90 mercury concentration system.

defined length of time over gold gauze forming an amalgam. After the required collection period, the mercury vapour is released by heating. The vapour is passed then to the spectrometer, producing a higher absorption signal than direct mercury vapour introduction. The manufacturer states that with control of the preconcentration process this system can detect mercury below 20 p.p.t.

Data handling

For improved **data analysis** in the Windows or UNIX environment StatSci offers S-Plus (*Reader Service No. 106*). This program utilizes a system called LEAP, which stands for 'look', 'explore', 'analyse' and 'present'. The look and explore functions allow the user to view data with a variety of graphics techniques and to perform dynamic three-dimensional visualization and other exploratory techniques, respectively. With 'analyse' the user may analyse with an object-oriented data language containing more than 1,650 classical and modern modelling, mathematical and graphics functions. With a simple click of the mouse, 'present' outputs what is on the screen with detailed, presentation-quality graphics that demonstrate the quality of analysis.

A new version of Windspeed software that lets **notebook PCs log and display data** is now available from Windmill software (*Reader Service No. 107*). Designed for a Windows format, this program continuously logs data to disk and charts them in real time. Users can then examine the collected data graphically, and cut out and save parts of interest in a choice of formats. Windspeed works with Microlink data acquisition hardware; these external units connect to a standard COM port on the computer, or to a PCMCIA network adapter. The system is



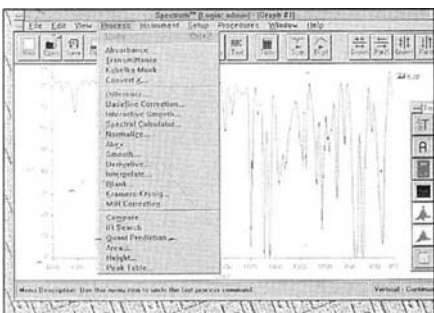
The LCQ LC/MS/MS from Finnigan.

capable of continuously streaming analog data to the hard disk at rates of up to 700 samples per second through the COM port, or 36,000 samples per second through Ethernet.

Infrared systems

Disposable **IR cards** from 3M are available in economical, 250-count packages from Spectra-Tech (*Reader Service No. 108*). These cards fit in the standard slide mount of spectrometers and are useful for qualitative IR transmission analysis, and only small volumes of sample are required to achieve optimum spectral results. The cards are disposable, which reduces the time and effort spent on clean-up. Type 61 cards are said to provide accurate readings from 4,000 to 1,300 cm^{-1} and to work especially well for substances requiring interpretation in the fingerprint region (1,600–400 cm^{-1}). However, for identifying samples in the 3,000 to 2,800 cm^{-1} range, the manufacturer recommends the type 62 IR card. These cards are effective from 4,000 to 1,300 cm^{-1} and are especially useful for samples requiring interpretation in the aliphatic C-H stretch region, and samples requiring temperatures up to 200 °C for short durations.

For **Fourier transform infrared (FT-IR) spectroscopy analysis** Perkin-Elmer offers validated Spectrum for Windows FT-IR software — designed to help users comply with good laboratory practice requirements (*Reader Service No. 109*). The new i-Series FT-IR microscope with infrared-microspectroscopy and automated, graphical-environment software provides users with the



Perkin-Elmer's FT-IR analysis software.

potential to use strictly defined rules formulated to check the qualitative and quantitative accuracy of spectra. It is designed to eliminate the need for manual checking procedures by automatically generating validation reports with validation of abscissa, ordinate, noise and ASTM0 (developed by the American Society for Testing and Materials for measuring the performance of FT-IR spectrometers).

Literature

Femtometrics offers a brochure that describes how proprietary surface acoustic wave (SAW) resonator technology has allowed the development of a sensitive **trace analysis instrumentation** that detects chemical vapours and air-

borne molecular contamination (*Reader Service No. 110*). Two products highlighted in the brochure are the NVR-200 real-time, non-volatile residue monitor and the SAWCVD 12A chemical-vapour, detection system. The NVR-200 model measures extremely low levels of airborne molecular contamination in cleanrooms and on critical surfaces, enabling users to identify contaminants that could have a negative impact on their production yields or operating performance.



SAW technology.

Melles Griot has published a complete, comprehensive **product guide for the photonics industry** (*Reader Service No. 111*). There are more than 1,200 pages containing new products, manufacturing capabilities and technical theory associated with optics, lasers, optomechanics and instruments.



Melles Griot guide to optics, lasers, optomechanics and instruments.

UV-vis spectroscopy

Hewlett-Packard has introduced the HP 8453 UV-visible spectroscopy systems (*Reader Service No. 112*). The systems include a spectrophotometer and application-specific, visible ChemStation software, which runs within a Windows environment. These new systems are designed to provide cost-effective and easy-to-use solutions to help analytical chemists and biochemists conform to the day-to-day requirements of good laboratory practice. Self-test procedures to check the electronics and key optical characteristics help to ensure consistent performance between verifications. An electronic log book records the results of self-tests, providing vital data for regulatory compliance.

Analytical Instrument Systems has announced three new **spectrophotometric instruments** that can be controlled from a notebook PC equipped with LabView executables and the DAQCard-700 data acquisition (DAQ) PCMCIA card from National Instruments (*Reader Service No. 113*). The DLK-1000 VIS and UV models are designed for applications in the visible (350–850 nm) and ultraviolet (185–450 nm) ranges. Users can run in single, multiple or continuous scan modes and can monitor up to six wavelengths simultaneously while recording cursor position to measure peak height and peak position. The VDIP-1 spectrometer is designed for portable applications — field scientists

should find this useful for measuring visible spectra in the field without using cuvettes or cumbersome power supplies. With the dip probe system, users can put the measuring probe (equivalent to a 1-cm path length) directly into a solution. The probe can then be rinsed and used to measure the next sample.

Mass measurements

Bruker Instruments has enhanced the Esquire **mass spectrometer** with an external ion source for electrospray, MALDI, LSIMS (liquid secondary ion mass spectroscopy) and other ionization techniques (*Reader Service No. 114*). The mass range of the instrument has been extended to m/z 6,000 — the high m/z detection with electrospray is important for many biochemists as it allows the detection of high molecular weight compounds with lower charge states. The instrument is said to have resolution sufficient to determine the charge state of multiple-charge ions from the ^{13}C isotopic spacing, which is useful for interpreting fragment ions from MS/MS spectra. With a standard resolution of 0.4 AMU at all masses, it now has an extended resolution mode over a smaller mass range so that ions with 3–5 charges are easily distinguished.

Meridian's FCD **time-of-flight mass spectrometer** with the FasTad integrating transient recorder allows operators to perform demanding gas chromatography analyses with improved efficiency (*Reader Service No. 115*). This system has fast spectral generation rates (up to 1,000 full mass range EI spectra per second) that are designed to enhance productivity, improve identification of unknowns, and make fast GC/MS possible. All spectra are said to be unskewed, even over the narrowest GC peaks, and represent true, reproducible fragmentation patterns that facilitate exact mass library searching. The manufacturer states that sensitivities are in the picogram range.

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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